CHARACTERIZATION OF *ZOOGLOEA RAMIGERA* IN BIOFILMS

By

Fuhua Lu

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE
UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1998

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to chairman of my supervisory committee, Dr. S. R. Farrah, for his support, guidance and commitment through the course of this research. My gratitude is also due to Dr. K. T. Shanmugam, Dr. B. L. Koopman, Dr. E. H. Hoffman and Dr. A. C. Wilkie for their guidance and counsel while serving on the advisory committee.

Thanks are extended to Dr. Chris West and Dr. Chasey Rentz for helping me conduct GC/MS analysis, Mr. Scott Whittaker and Ms. Karen Vaughn of the University of Florida ICBR Core Laboratory for their assistant in preparation and examination of SEM samples, and Ms. Sandy Fisher of University of Florida Lake Watch for providing information on lakes. Special thanks to all colleagues in Dr Farrah's lab, Jerzy Lukasik, Haitao Zhou, Mike Burne Jr., Troy Scott and Cheryl Boice, for their friendship and encouragement. I would like to extend my thanks to Engineering Research Center (ERC) professors and colleagues, for their support and participation during the course of this research.

I also would like to thank Scott M. Buntin for his encouragement. Finally, I wish to express sincere gratitude to my father, my mother, my brother and my sister, for their unconditional love, understanding and patience.

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LIST OF ABBREVIATIONS

AEC	Aminoethyl carbozole
AHL	Acetylated homoserine lactones
BOD	Biological oxygen demand
BSA	Bovine serum albumin
CTAB	Cetyltrimethylammonium bromide
DABCO	1,4-diazabicyclo (2,2,2) octane
DEPC	Diethyl pyrocarbonate
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
GC/MS	Gas chromatography / mass spectrometry
HMDS	Hexamethyldisilizane
MIC	Minimal inhibitory concentration
MINT	Malachite green - 2(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride
MPN	Most probable number
PFA	Paraformaldehyde

PBS	Phosphate buffered saline
RNase	Ribonuclease
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase-polymerase chain reaction
SEM	Scanning electron microscopy
TBS	Tris buffered saline
TMS	Tetramethylsilane
Tris	Tris-(hydroxymethyl)-aminomethane
UV	Ultraviolet
YP	Yeast extract - peptone

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF *ZOOGLOEA RAMIGERA* IN BIOFILMS

By

Fuhua Lu

December, 1998

Chairman: Dr. Samuel R. Farrah
Major Department: Microbiology and Cell Science

Zoogloea ramigera is an extracellular polymer-producing bacterium and forms flocs which include typical finger-like projections and amorphous flocs. Scanning electron microscopy (SEM) indicated that cells used the extracellular polymer as attachment sites and were embedded within the extracellular polymer. Finger-like projections which were similar to those from laboratory cultures of *Z. ramigera* were also found on the surface of sand which had raw sewage supplemented with phenol passed through them. *Z. ramigera* was isolated from raw sewage, mixed liquor suspended solids and lake water. The efficiency of *Z. ramigera* isolation was greatly increased by using m-toluic acid isolation medium combined with 1 μg/ml trimethoprim, and/or 10 μg/ml sulfadiazine.

Indirect immunoassay methods for the detection of *Z. ramigera* were developed using polyclonal antibodies against the cells or the isolated extracellular polymer of the

neotype *Z. ramigera* strain 106 (ATCC 19544). The use of goat anti-rabbit IgG conjugated with FITC or biotin or colloidal gold as the secondary antibody allowed detection of *Z. ramigera* in environmental samples. These methods were also used as part of a most probable number (MPN) procedure to quantitate *Z. ramigera* at different stages of the wastewater treatment processes as well as in different lakes. It was found that the cells and the extracellular polymer of naturally occurring zoogloeal projections are antigenically and structurally related to those of *Z. ramigera* 106. *Z. ramigera* could be found in all stages of wastewater treatment processes, eutrophic lakes, mesotrophic lakes, and some oligotrophic lakes. The highest concentration of *Z. ramigera* was found in the mixed liquor stage of the two wastewater treatment plants in Gainesville, Florida.

By using bacterial 16S rRNA as template, and the primers specific for *Z. ramigera* 106 16S rRNA, RT-PCR was also used to identify *Z. ramigera* in biofilms from natural environments.

Zeta potential measurement indicated that overall surface charge of *Z. ramigera* was negative. Gas chromatography-mass spectrometry (GC/MS) analysis indicated the predominance of carbohydrate, especially amino sugars in the extracellular polymer produced by *Z. ramigera*. The use of MINT test following treatment of chlorine indicated that the extracellular polymer protected *Z. ramigera* cells from chlorine inactivation. This is true for *Z. ramigera* in both biofilms from natural environments and laboratory cultures.

Biofilms and other organic and inorganic materials in wastewater blocked positively charged sites on the surface of aluminum hydroxides coated sand. This loss of

electropositive charge character was found to correspond to the decrease in removal efficiency of the two bacteriophages, which further supports the importance of electrostatic forces in virus-sand interaction.

INTRODUCTION

Zoogloea ramigera

Finger-like zoogloeal projections were observed among decaying algae and the bacteria within the projections were named *Zoogloea ramigera* by Itzigsohn (1868). Since then, finger-like zoogloeal projections have been observed in trickling filter slime layers, and in association with solids formed during aerobic treatment of wastewater (Bitton, 1994; Butterfield, 1935; Farrah and Unz, 1975; Rossello-mora et al., 1995; Unz and Dondero, 1967a; Unz and Farrah, 1976a). In some cases, bacteria associated with these samples were isolated and studied in pure culture (Butterfield, 1935; Unz and Dondero, 1967a,b). Since the term "finger-like" projection was subjected to different interpretations, bacteria with different morphological and chemical characteristics were considered to be *Z. ramigera* (Butterfield, 1935; Crabtree et al., 1965; Friedman et al., 1968; Unz, 1971; Unz and Dondero, 1967b). Studies by comparing 16S rRNA sequence and chemotaxonomic properties have shown that *Z. ramigera* 106 (ATCC 19544) is related to the bacteria within natural zoogloeal projections but not to the other bacteria that were previously termed *Z. ramigera* (Rossello-mora et al., 1993; Shin et al., 1993). Recently, one of the previously misclassified *Zoogloea* species was reclassified as *Duganella zoogloeoides* (Hiraishi et al., 1997).

It has been difficult to isolate *Z. ramigera* from natural environments. Different isolation procedures have been tried such as dispersion dilution method (Butterfield, 1935) and micro manipulation method (Unz and Dondero, 1967a). Effect of different media on isolation was also investigated (Dugan and Lundgren, 1960). Since *Z. ramigera* grows slowly, often resulting in overgrowth by other microorganisms, conventional bacterial isolation techniques were not very effective for rapid isolation until aromatic compounds such as benzoate, m-toluic acid, phenol and cresol were incorporated into basal media as a carbon source (Unz and Farrah, 1972).

Wastewater Treatment

Since the proper settling of solids produced during aerobic wastewater treatment is important to produce clear effluents, many researchers have studied the microorganisms that affect this process. This settling requires a proper balance of floc-forming bacteria and filamentous bacteria (Bitton, 1994; Nozawa et al., 1987). If the numbers or activities of the floc-forming bacteria are reduced, sludge bulking may occur, and turbid rather than clear effluents will be produced. Because of its observed association with sludge flocs, *Z. ramigera* has been regarded as an important bacterium in the activated sludge treatment process (Bitton,1994). In one study, the ability to settle bulking sludge was restored by seeding the sludge with *Z. ramigera* and other bacteria (Nozawa et al., 1987).

Similarly, in trickling filters, the slime layer that develops on the support media is considered to be very important for the colonization of bacteria (Bitton, 1994). This

slime layer is an extensive polysaccharide matrix that is generally referred to as the glycocalyx. This glycocalyx anchors the bacteria and helps in the removal of complex organic and inorganic materials from wastewater (Bitton, 1994). *Z. ramigera* has been isolated from finger-like projections obtained from trickling filter slimes and is thought to be an essential player in the filtration process (Butterfield, 1935; Unz and Dondero, 1967a).

Although *Z. ramigera* has been thought to be important in wastewater treatment, there is little information on the distribution and concentration of this organism in wastewater treatment processes. Several procedures for the detection of *Z. ramigera* in natural samples have been established and used to study *Z. ramigera* in wastewater samples, including using fluorescein-conjugated antibody against *Z. ramigera* cells and fluorescein-labeled 16S rRNA oliogonucleotide probe (Rossello-mora et al., 1995; Farrah and Unz, 1975).

Williams and Unz (1983) used enrichment procedures to support the development of finger-like *Zoogloea*. These authors found that bacteria capable of producing finger-like *Zoogloea* were less than 0.01% of the total microbial population in mixed liquor solids. However, Rossello-Mora et al.(1995) found that up to 10% of the bacteria within activated sludge flocs reacted to the fluorescein-labeled oligonucleotide probe complementary to the 16S rRNA of *Z. ramigera* (ATCC 19544). Unz and Farrah(1976a) showed that finger-like zoogloeae were not usually observed in mixed liquor suspended solids but could develop from the flocs under the proper incubation conditions.

Z. ramigera Extracellular Polymer

Extensive studies have characterized the *Z. ramigera* extracellular polymer. It was indicated that no protein or ether-soluble material was detected and amino sugars are the principal constituent after acid hydrolysis of extracellular polymer (Farrah and Unz, 1976). Separation of hydrolyzed extracellular polymer of *Z. ramigera* isolated from activated sludge by paper and ion-exchange chromatography suggested that amino sugars might be glucosamine and fucosamine and the ratio of the two amino sugars was between 1:1.5 to 1:2 (Tezuka, 1973). Amino sugars have also been found in an extracellular polymer produced by other bacteria. In *Streptococcus pneumoniae*, the extracellular polymer contains a tetrasaccharide repeating unit, three different amino sugars, N-acetyl-D-mannosamine, N-acetyl-L-fucosamine and N-acetyl-D-galactosamine, are sequentially linked to a D-galactopyranosyl residue carrying a 2,3-linked pyruvate ketal (Jansson et al., 1981).

Biofilms

Surfaces exposed to a variety of types of water are found to develop biofilms of microorganisms. Biofilm bacteria live in a complex microbial community that has primitive homeostasis, a primitive circulatory system and metabolic cooperativity so that each of these sessile cells reacts to special environment fundamentally different from planktonic counterparts (Costerton et al, 1995; Kolter and Losick, 1998). Biofilm microorganisms are usually more resistant to environmental stress and antimicrobial agents than planktonic counterparts.

Besides the difference between biofilm microorganisms and planktonic microorganisms, the precise manner by which extracellular polymer protects the cells is unclear, but the presence of bound extracellular enzymes, such as β-lactamase, within the *Pseudomonas aeruginosa* glycocalyx may reinforce its action as a diffusion barrier (Bolister et al., 1991) with respect to some antibiotics, and its molecular severing properties are enhanced through binding of divalent cations, such as calcium, from the environment (Hoyle et al., 1992). It has also been proposed that the glycocalyx provides intrinsic protective effects against antimicrobial agents which are additional to those associated with its diffusion and charge-related properties (Hodges and Gordon, 1991). For example, *Pseudomonas aeruginosa* povidone-iodine resistance seems to be due to the protective layering of cells within the glycocalyx which increase the time required for iodine to contact cells in the deepest layers of the biofilm (Brown et al., 1995). The properties of biofilms have been considered in developing methods to control microbial biofilm growth (Wood et al., 1996).

Chlorine Disinfection

In wastewater treatment processes, chlorine is a commonly used disinfectant to inactivate bacteria and viruses (Bitton, 1994). The mechanisms of chlorine inactivation were extensively investigated (Bitton and Koopman, 1982; Costerton et al., 1995; De Beer et al., 1994; Dutton et al., 1983; Herson et al., 1987; Huang et al., 1995; LeChevallier et al., 1988a,b; LeChevallier et al., 1984) so that strategies could be developed to effectively control water quality and prevent waterborne disease outbreaks.

Besides, chlorine was also used to control sludge bulking which results from a predominance of filamentous bacteria and absence of floc forming bacteria during activated sludge process (Bitton and Koopman, 1982). It was noticed that bacteria attached to surfaces (Brown et al., 1995; De Beer et al., 1994) or extracellular-polysaccharide-coated bacteria (Bolister et al, 1991) were more resistant to antibacterial agents (including chlorine) than were their planktonic counterparts. It was suggested that the reason for reduced efficacy of chlorine against biofilm bacteria as compared with its action against planktonic cells might be the limited penetration of chlorine into the biofilm matrix (De Beer et al., 1994; Herson et al., 1987; Huang et al., 1995; LeChevallier et al., 1988a,b; LeChevallier et al., 1984).

Filtration Process in Wastewater Treatment

Filtration processes are used in both water and wastewater treatment. In wastewater treatment process, trickling filters are used in aerobic treatment of wastewater. These filters consist of inanimate materials such as rocks. Wastewater is passed over these filters to allow biofilm developed on the surface of the filter, thus to reduce the level of organic contaminants in wastewater. This filtration process has been used to remove pathogenic bacteria and virus from water and wastewater. It was indicated that biofilm development on filter media enhanced physical entrapment of bacteria and bacteria-sized fine particles from water (Banks and Bryers, 1992; Drury et al., 1993; Rittmann and Wirtel, 1991; Sprouse and Rittmann, 1990; Schuler et al., 1991).

Sand filters are also used in water and wastewater treatment. It has been shown that surface modification of filter media with various metal oxides, peroxides or hydroxides also increased the microorganism removal efficiency by changing the surface charge of the media from electronegative to electropositive, thus decreasing the electrostatic repulsion between the particles and the adsorbing solid (Lukasik et al., 1996; Truesdail et al., 1998).

Objectives

The objectives of this study were (1) to study the structure of natural and laboratory finger-like projections; (2) to improve the *Z. ramigera* isolation method; (3) to develop *Z. ramigera* detection methods by using immunological and molecular procedures; (4) to use these procedures to estimate the number of *Z. ramigera* in wastewater at different stages of treatment and in lake water; (5) to characterize the extracellular polymer of *Z. ramigera* and its influence on chlorine inactivation; and (6) to evaluate the effect of biofilm and other organic and inorganic materials in wastewater on virus removal by aluminum hydroxide coated sand.

LITERATURE REVIEW

Zoogloea ramigera

Z. ramigera is an extracellular polymer producing bacterium that forms typical finger-like projections and is found among decaying algae, in wastewater, and in other organically enriched environments. The bacteria within the finger-like zoogloeal projections were named *Zoogloea ramigera* by Itzigsohn (1868). *Z. ramigera* is a gram-negative, aerobic, chemoorganotrophic bacterium. It also grows anaerobically in the presence of nitrate (nitrate respiration) and denitrification occurs with formation of N_2. Major carbon sources include lactate, glutamate, alcohol, benzonate, and m-toluate. Benzene derivatives are used by *meta* cleavage (Holt et al., 1994). Neither acid nor gas is produced from carbohydrates (Butterfield, 1935; Heukelekian and Littman, 1939; Unz and Dondero, 1967b). It might be that the bacterium either does not attack the carbohydrates to produce acid by-products from them, or produces alkaline materials from the proteins which neutralize the acids produced or further metabolizes the acids as rapidly as they are formed (Heukelekian and Littman, 1939). Optimum temperature and pH for growth are near 28°C and pH 7.0, respectively (Unz and Dondero, 1967b). The extracellular polymer produced by *Z. ramigera* attaches to its cell walls and does not make broth viscous during the synthesis phase. This property allows high oxygen transfer rates to be maintained during the high oxygen demand period. Furthermore, very low

oxygen consumption was observed during the period of polysaccharide release when the oxygen transfer rate can not be raised without very high energy input (Norberg and Enfors, 1982). The extracellular polymer production is also influenced by the carbon and nitrogen sources (Unz and Farrah, 1976b).

Z. ramigera was originally observed among decaying algae (Itzigsohon, 1868). Later, the recognition of the possible importance of *Z. ramigera* in activated sludge and trickling filter have led to extensive studies of *Z. ramigera* (Crabtree et al., 1965). The dispersion dilution method (Butterfield, 1935), micro manipulation (Unz and Dondero, 1967a) and the use of isolation media containing aromatic compounds (Unz and Farrah, 1972) were used to isolate *Z. ramigera* from natural environments such as activated sludge and trickling filter slime. The effects of different media on isolation were also investigated (Dugan and Lundgren, 1960).

Biochemical tests of *Z. ramigera* allowed convenient identification of *Z. ramigera* from natural environments (Unz, 1971). It was found that *Z. ramigera* isolated from activated sludge and trickling filter functions similarly (Wattie, 1942). Fluorescence microscopy (Farrah and Unz, 1975) and scanning electron microscopy (SEM) (Sich and Van Rijn, 1997) were used to observe the presence of *Z. ramigera* in natural environments. In order to understand the mechanism of *Z. ramigera* function in activated sludge and trickling filter, structure and composition of extracellular polymer surrounding *Z. ramigera* have also been investigated (Crabtree et al., 1966; Friedman et al., 1968; Horan and Eccles, 1986; Norberg and Enfors, 1982; Parsons and Dugan, 1971; Unz and Farrah, 1976b).

Based on the physiology and biochemical properties of *Z. ramigera* (Heulelekian and Littman, 1939; McKinney and Harwood, 1952; Unz and Dondero, 1967b; Krul, 1977), efforts were made to establish its identification criterion and generic status (Zvirbulis and Hatt, 1967; Crabtree and McCoy, 1967; Munich, 1979; Skerman et al., 1980; Rossello-mora et al., 1993; Shin et al., 1993). It was first requested that ATCC 19623 (strain I-16-M) should be accepted as the neotype strain in 1967 (Crabtree and McCoy, 1967). In 1971, ATCC 19544 (strain 106) was suggested as the neotype strain because I-16-M did not form typical finger-like projection (Unz, 1971) and no extracellular material was observable around I-16-M (Friedman et al., 1968). However, based on the observation of floc formation during growth, three phylogenetically distantly related strains, ATCC 19544^T (strain 106) (T=type strain), ATCC 25935 (strain 115), and ATCC 19623 (strain I-16-M), were included in the same species. Through chemotaxonomic study (mainly polyamine and quinone composition) and comparative analyses of 16S rRNA primary structure, it has been suggested that only isolates that clearly resemble neotype strain ATCC 19544^T phenotypically should be considered genuine members of *Z. ramigera* (Rossello-mora et al., 1993). In fact, 16S rRNA sequence comparisons and distance matrix tree analysis revealed that *Z. ramigera* 106 forms a lineage with *Rhodocyclus purpureus* in the beta subclass of *proteobacteria.* ATCC 25935 was shown to belong to the beta subclass of the class *Proteobacteria* with members of the genus *Telluria* as its closest relatives. In contrast, ATCC 19623 proved to be a member of the alpha subclass of the proteobacteria, closely related to

Agrobacterium tumefaciens. (Shin et al., 1993). Recently, ATCC 25935 was reclassified as *Duganella zoogloeoides* (Hiraishi et al., 1997).

The possible biotechnological importance of *Z. ramigera* is that it plays important roles in wastewater treatment process, especially in flocculation of activated sludge and biofilm formation on the surface of biofilm reactors.

Overview of Wastewater Treatment Process

The objectives of wastewater treatment processes are reduction of organic content (BOD), nutrients (N, P) and removal/reduction of pathogenic microorganisms and parasites. The conventional wastewater treatment process includes (1) preliminary treatment to remove debris or coarse materials; (2) primary treatment which is by physical means such as screening and sedimentation; (3) secondary treatment which is by biological means such as activated sludge, trickling filter, or oxidation ponds, and chemical means such as disinfection; and (4) tertiary or advanced treatment which is mainly by chemical means, such as flocculation, filtration, and disinfection.

Influent from a collection system or pumping station is first treated by preliminary processes (pumping, screening and grit removal) and primary settling to remove heavy solids and floatable materials. Primary solids may go to landfills. Primary effluent is treated by biological means (eg. activated sludge or trickling filter). If tricking filters are used, the primary effluent is applied to filter beds containing natural materials (rock, coal) or synthetic (plastic) supports that permit biofilm development. Then, sludge (solid sloughed off the filters) and liquids are separated in settling tanks. In the activated

sludge process, primary effluent is mixed with returned activated sludge (RAS) to form the mixed liquor in the aeration tank where aeration is provided by mechanical means. When treatment with activated sludge is complete, the mixture goes to sedimentation tanks to separate solids (sludge) and liquid. A portion of sludge is recycled to provide an inoculum for the influent sewage. The rest of sludge is usually further processed by screening, thickening, dewatering, conditioning, and stabilization (anaerobic digestion, aerobic digestion, composting, lime stabilization and heat treatment) before land application. The secondary effluent is further treated by chlorination, filtration, flocculation, and so on before agriculture reuse, landscape irrigation, ground water recharge, recreational reuse, nonpotable urban reuse, potable reuse, industrial reuse, or released to receiving waters (Fig. 1).

BOD, N and P are greatly reduced during biological wastewater treatment processes. They are also reduced by chemical means such as flocculation, composting and lime stabilization. Efficient microorganism removal from wastewater is very important in order to prevent waterborne disease outbreaks and to protect the public's health. During primary and secondary wastewater treatment, most of the protozoans are settled with sludge solid due to their bigger size compared with bacteria and viruses. Many bacteria and viruses associated with sludge solids are also settled with the sludge. Almost 90% of viruses may be removed from water by the activated sludge treatment process (Rao and Melnick, 1986). It was reported that *Z. ramigera* extracellular polymer avidly adsorbed ^{125}I-labeled polio virus and either precipitated the virions or neutralized them (Smith, 1983; Rao and Melnick, 1986).

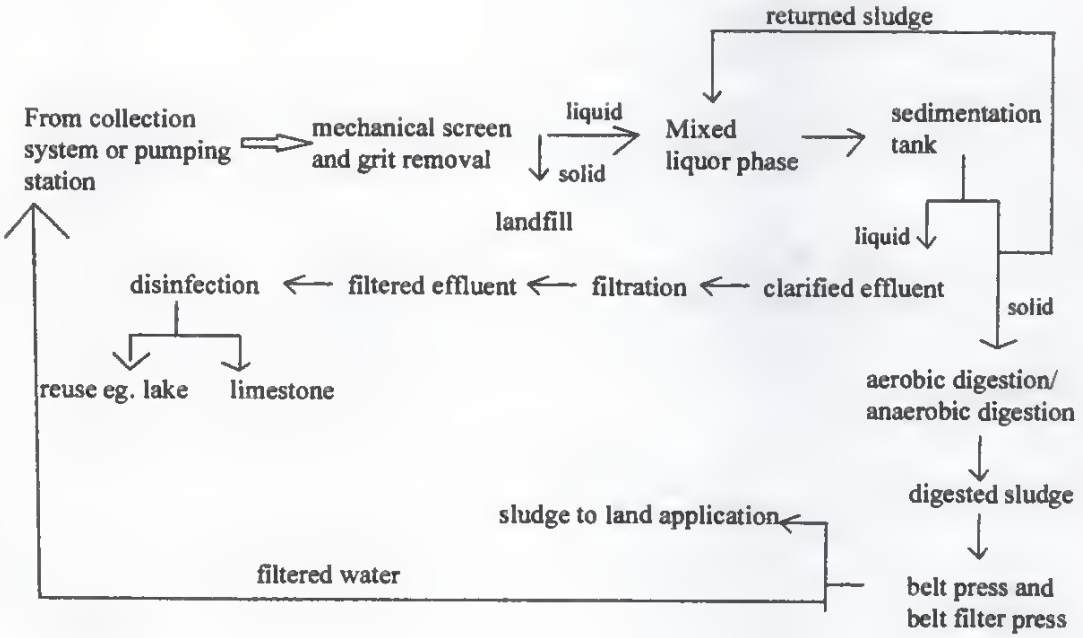

Fig. 1. Conventional wastewater treatment process

Ciliated protozoans are the predominant protozoans present in activated sludge. They consume many of the absorbed materials in sludge, including viruses and bacteria (Rao and Melnick, 1986; Bitton, 1994). These microorganisms in the sludge are reduced by sludge processes such as composting, heat treatment, aerobic/anaerobic digestion, and so on. The microorganisms in the liquid phase of wastewater are removed by flocculation and disinfection treatments such as with chlorine, ozone, chlorine dioxide, and so on. The order of removal efficiency by disinfectants usually is bacteria > virus > protozoan.

Besides disinfection treatment, filtration has been a useful method for removal of bacteria, virus and other fine particles from wastewater during tertiary wastewater treatment. Rapid sand filtration and biofilm-mediated slow sand filters have been used for removal of bacteria and viruses in wastewater treatment plants. In order to improve the removal of microorganisms from water and wastewater, the mechanisms of microorganism adsorption to solid particles have been investigated (Mills, et al., 1994; Gerba., 1984). It was indicated that electrostatic interactions are important for filtration of recombinant Norwalk virus particles and bacteriophage MS2 in quartz sand (Redman et al., 1997). Hydrophobic interactions (Bales and Li, 1993), van der Waals forces, and surface properties such as surface roughness and surface charge heterogeneity are also important in microorganisms-solid interaction (Lukasik et al., 1996; Truesdail et al., 1998).

Different modifications of filter media have been tried, including the use of metal peroxide (Asghari and Farrah, 1993; Farrah and Preston, 1991; Gerba et al., 1988), metal oxide (Stenkamp and Benjamin, 1995), and metal hydroxide coatings (Farrah and

Preston, 1985; Lukasik et al., 1996; Lukasik et al., 1998). Such modifications have increased the removal of microorganisms from water relative to untreated granular media. The reason is that modifying the sand or diatomaceous earth with various metal oxide, peroxide or hydroxide surface coatings changed the surface charge from electronegative to electropositive, thus decreasing the electrostatic repulsion between the particles and the adsorbing solid. Therefore, these modified filter media are promising materials to be used in removing microorganisms in wastewater filtration processes.

When the modified filter media are used in wastewater treatment plants, the development of biofilms or the presence of organic and inorganic materials in the wastewater have the potential to change the surface properties of the filter media, and thus influence the microorganism removal capacity. It has been suggested that biofilm development on slow sand filters (Schuler et al., 1991), glass, polycarbonate and granular activated carbon surfaces (Banks and Bryers., 1992; Drury et al., 1993; Rittmann and Wirtel., 1991; Sprouse and Rittmann., 1990) enhanced the physical entrapment of bacteria and bacterium-sized fine particles. However, it was also found that sewage-derived organic matter blocked the attachment sites on ferric oxyhydroxide-coated quartz thus decreasing bacteriophage PRD1 adsorption (Pieper et al., 1997).

Overall, biological wastewater treatment processes are important for microorganism removal as well as reduction of organic materials. It has long been believed that *Z. ramigera* plays important roles in wastewater treatment processes such as activated sludge flocculation and biofilm formation on the surface of trickling filters.

The Function of *Z. ramigera* in Activated Sludge

In the activated sludge process, wastewater is fed continuously into an aerated tank, where the microorganisms metabolize the organic materials. Biological flocs form during this process. These flocs consist of a variety of microorganisms and are collectively referred to as activated sludge. Following the treatment, a portion of the sludge is discarded (wasted) and the rest is returned to the aeration tank. The relatively clear supernatant from the final settling tank is the secondary effluent.

The primary feeders in activated sludge are bacteria. Secondary feeders are holozoic protozoans. Microbial growth in the mixed liquor is maintained in the declining or endogenous growth phase to ensure good settling characteristics. Activated sludge is truly an aerobic treatment process since the biological floc is suspended in liquid media containing dissolved oxygen. Dissolved oxygen extracted from the mixed liquor is replenished by air supplied to the aeration tank (Viessman and Hammer, 1985).

In activated sludge, extracellular polymer produced by *Z. ramigera* and other activated-sludge microorganisms plays a role in bacteria flocculation and floc formation processes which are essential prerequisites for the efficient and economical operation of an activated-sludge wastewater treatment plant (Bitton, 1994). Floc formation during the aeration phase is also instrumental in removing undesirable microorganisms.

The proper settling of activated-sludge solid requires a proper balance of floc-forming bacteria and filamentous bacteria. If the numbers or activities of the floc-forming bacteria are reduced, sludge bulking may occur and turbid rather than clear effluents will be produced (Bitton, 1994; Nozawa et al., 1987). Sludge bulking is one of

the major problems affecting biological wastewater treatment. There are several approaches for controlling sludge bulking, including addition of oxidants such as chlorine or hydrogen peroxide, flocculent such as synthetic organic polymers, lime and iron salts, and using biological selectors (Bitton, 1994). It was indicated that the ability to settle bulking sludge was also restored by seeding sludge with *Z. ramigera* (Nozawa et al., 1987).

Farrah and Unz (1976) studied *Z. ramigera* extracellular polymer and found that amino sugars are the principal constituent after acid hydrolyzation of extracellular polymer and the amino sugars content in extracellular polymer isolated from activated sludge flocs was similar to that of from *Z. ramigera*. Paper and ion-exchange chromatography separation of hydrolyzed extracellular polymer of *Zoogloea* isolated from activated sludge suggested that the amino sugars are glucosamine and fucosamine (Tezuka, 1973). The isolated polymer from activated-sludge microorganisms has been found to contain neutral sugars, amino sugars, uronic acids and amino acids, which indicates their heteropolysaccharidic character (Hejlar and Chudoba, 1986). The molecular weight of the extracellular polymer fraction ranged from $3x10^5$ to $2x10^6$ Daltons. Glucose, galactose, mannose, glucuronic acid and galacturonic acid were detected in this fraction (Horan and Eccles, 1986).

Therefore, there are negative and positive charges in the extracellular polymer which allow the polysaccharide to behave as polyelectrolyte. It is observed that extracellular polymers produced by microorganisms commonly found in activated sludge display a great affinity for metals. Several types of bacteria (e.g. *Z. ramigera, Bacillus*

lichenifomis), some of which have been isolated from activated sludge, produce extracellular polymers that are able to complex and subsequently accumulate metals, such as iron, cooper, cadmium, nickel or uranium. For example, *Duganella zoogloeoides* (ATCC25935) can accumulate up to 0.17 g of Cu per gram of biomass (Norberg and Persson,1984; Norberg and Rydin, 1984). This bacterium, when immobilized in alginate beads, is also able to concentrate cadmium to as high as 250 mg/l (the alginate beads also absorb some of the cadmium) (Kuhn and Pfister, 1990). The heavy metal adsorption of *Duganella zoogloeoides* (ATCC25935) might be related to its production of an acidic extracellular polymer such as succinoglycan which contains glucose, succinate and pyruvate (Ikeda et al., 1982). Correlation between highly anionic charged polymers and metal complexing capacity was found by study of chelating properties of extracellular polysaccharide produced by *Chlorella* spp. (Kaplan et al., 1987).

It is also noticed that the production of extracellular polymer by bacteria may drastically reduce the saturated hydraulic conductivity of sand columns (HCsat) and this effect was only observed when the extracellular polymer produced in the form of loose slime layers. Cell-bound capsular extracellular polymer had no significant effect on the HCsat (Vandevivere and Baveye, 1992).

The Function of *Z. ramigera* During Biofilm Formation

Biofilm formation is thought to result from the concerted action of primary attachment to a specific surface and accumulation in multilayered cell clusters. Biofilms are defined as matrix-enclosed bacterial populations adherent to each other and/or to

surfaces or interfaces. This definition includes microbial aggregates and floccule and also adherent populations within the pore spaces of porous media (Costerton et al., 1995). Biofilm is ubiquitous. It exists in natural environments (Gillan et al., 1998; McLean et al., 1997), and in clinical settings (Stickler et al., 1998). The structural and physiological heterogeneity of biofilm is now widely recognized (Huang et al., 1995; Huang et al., 1998; Stewart et al., 1997; Xu et al., 1998).

In addition to traditional methods (cultivation and/or microscopy), genetic methods are also available to characterize microbial diversity. Amplification and sequence analysis of the 16S rRNA (rDNA) has been successfully used for understanding the biology of microbial community (Britschgi and Givonnoni, 1991; DeLong et al., 1993; Gillan et al., 1998; Liesack and Stackebrandt, 1992; Ward et al., 1990). In one study, the genetic diversity and phylogenetic affiliation of biofilm bacteria which covered the shell of bivalve *Montecuta ferruginosa* were determined by denaturing gradient gel electrophoresis (DGGE) analysis of 16S ribosomal DNA PCR products obtained with primers specific for the domain *Bacteria* (Gillan et al., 1998). Fluorescein *in situ* hybridization (FISH) by using fluorescein labeled oligonucleotide probes are also frequently used to detect and characterize bacteria in microbial community (Muyzer and Ramsing, 1995).

During biofilm formation, initial attachment requires flagella or surface adhensins and nutritional signal from environments (Costerton et al., 1995). Attached bacteria excrete extracellular polymer as a matrix for biofilms (Costerton et al., 1987; Allison and Sutherland, 1987). Mature biofilms form mushroom- and pillar-like

structures with water channel between them, which function much like primitive circulatory system (Costerton et al., 1995). It has been noticed that bacterial cells in biofilm are usually more resistant to environmental stress and antibacterial agents than planktonic cells (Allison and Sutherland, 1987; Marshall et al., 1989; Ophir and Gutnick, 1994; Brown et al., 1995). It is said that bacterial adhesion might trigger the expression of a sigma factor which regulates a large amount of genes so that biofilm cells are phenotypically distinct from planktonic cells of the same species (Costerton et al., 1995; Yu and Mcfeters, 1994). Recent study indicated that the pattern of gene expression within biofilm is largely controlled by the metabolic activity of the microorganisms and the local availability of carbon and energy sources (Huang et al., 1998; Xu et al., 1998).

It has been noticed that biofilm formation involves cell-to-cell signals (Passador et al., 1993; Kolter and Losick, 1998; Davies et al., 1998). Acylated homoserine lactones (AHLs) are chemical signals that mediate population density-dependent (quorum-sensing) gene expression in numerous Gram-negative bacteria (Stickler et al., 1998). Structures of signals in *Pseudomonas aeruginosa* are N-3-(oxooctanoyl)-L-homoserine lactones and N-(butyryl)-L-homoserine lactones. These signals were required for the expression of the virulence factors toxin A and elastase (Passador et al., 1993).

AHLs accumulated in bacterial cultures as membrane-permeant signal molecules. At a threshold population density, the accumulated AHLs interact with cellular receptors controlling the expression of a set of specific target genes which respond to local cell density (Fuqua et al., 1996; Salmond et al., 1995; Stickler et al., 1998). Therefore, AHLs are important in the development of the biofilm-specific physiology (Heyes et al., 1997).

For example, a *Pseudomonas aeruginosa* mutant strain unable to make AHLs did not produce a typical biofilm and was sensitive to the biocide sodium dodecyl sulfate (Davies et al., 1998). It was indicated that AHLs are not only produced in natural biofilms growing on submerged stones taken from the San Marcos river in Texas (McLean et al., 1997), but also produced by biofilm in clinical setting such as indwelling urethral catheter (Stickler et al., 1998).

Analogues of AHLs capable of interfering with signaling have the potential to be used to prevent the formation and development of biofilm on implanted medical devices. It was indicated the furanone derivatives produced by the seaweed *Delisa pulchra* inhibit swarming of *Serratia liquefaciens* which is AHLs-regulated. It was speculated that furanone derivatives mimic AHLs signaling process by blocking transcriptional activation of target genes (de Nys et al., 1995; Erble et al., 1996; Givskov et al., 1996). Therefore, it is very likely to be able to control biofilm formation and dissolution *in situ* by using AHLs and its analogues.

Extracellular polymer production by biofilm bacteria not only helps the initial attachment of bacteria to surfaces but also helps the formation and maintenance of micro colonies and biofilm structure, enhances biofilm resistance to environmental stress and antimicrobial agents, protects bacteria in the biofilm from protozoan grazing and provides biofilm nutrition (Allison and Sutherland, 1987; Heissenberger et al, 1996; Marshall et al.,1989; Ophir and Gutnick, 1994). The extracellular polymer in biofilm is also highly heterogenous and has been demonstrated *in situ* to vary spatially, chemically and physically (Lawrence et al., 1994; Wolfaardt et al., 1993; Wolfaardt et al., 1994). In

addition, the chemically reactive extracellular polymer is generally the first biofilm structure to come in contact with potential substrates, predators, antimicrobial agents/antibiotics and other bacteria, and thus is of considerable applied and ecological importance. For example, bacterial cells would be attached to the organic nutrients that concentrate naturally at surfaces in aquatic systems, and the extracellular polymer that mediate their adhesion to surfaces would further concentrate dissolved organic molecules and cations out of bulk fluid.

Biofilm Reactors

In wastewater treatment plants, biofilm reactors include trickling filters, rotating biological contractors (RBC), and submerged filters (down-flow and up-flow filters). These reactors are used for oxidation of organic matter, nitrification, denitrification or anaerobic digestion of wastewater. During biofilm formation on trickling filter surfaces, the surface of the support materials is colonized with Gram-negative bacteria followed by filamentous bacteria. There are two steps in the absorption of bacteria to biofilm surfaces. The first step is reversible sorption, mainly controlled by electrostatic interactions between absorbent and the cells. The second step consists of irreversible absorption of cells, resulting from the formation of polysaccharide-containing matrix, named glycocalyx. Glycocalyx not only helps anchor the biofilm microorganisms to the surface, but also helps protect microorganisms from predation and from chemical insult. There are also polyanionic compounds in glycocalyx that complex the metal ions (Bitton, 1994).

Z. ramigera was found in biofilm during denitrification by fluidished bed reactors (Sich and Rijn, 1997). It degraded phenols and nitrogen-containing aromatic compounds (Koch et al., 1991) and co-existed with *Pseudomonas* during these processes. Initial colonization on granules was mainly *Zoogloea* species. During the period of co-existence, *Zoogloea* cells provided a setting substrate for *Pseudomonas* and the gelatinous matrix provided by *Zoogloea* might have served as nutrient trap for *Pseudomonas* which eventually covered the entire outer layers of the granules (Sich and Rijn, 1997).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for Microorganism Detection

In addition to traditional microbiological and immunological methods for microorganism detection, molecular procedures have been increasingly popular and more frequently used to detect virus, bacteria and protozoan in clinical and environmental samples.

Comparing with immunological methods (eg. immunohistology, ELISA, etc) and tissue culture, RT-PCR has been extensively used as a sensitive, specific and time saving method to detect genes of RNA viruses (Hayase and Tobita, 1998), including dengue virus (Chow et al., 1998; Hober et al., 1998; Liu et al., 1997), hepatitis A virus (Cromeans et al., 1997), hepatitis C virus (Jeannel, et al., 1998; Laursen et al., 1998; Whitby and Garson, 1997), citrus psorosis virus (Barthe et al., 1998), reovirus (Tyler et al., 1998), enterovirus (Chung et al., 1996; Gantzer, et al., 1997), respiratory virus

(Rohwdder et al., 1998; Valassina et al., 1997), measles virus (Chadwick et al., 1998; Kawashima et al., 1996), mumps virus (Kashiwagi et al., 1997; Cusi et al., 1996), HIV (Beilke et al., 1998; Contoreggi et al., 1997), and so on.

Several varieties of RT-PCR was developed for virus detection. For example, multiplex RT-PCR was used to rapidly detect and identify different serotype of viruses such as human parainfluenza viruses 1, 2 and 3 (Echevarria et al., 1998), or different species such as influenza A virus (IA) and respiratory syncytial virus (RS) (Valassina et al., 1997). Nested or semi-nested RT-PCR can be used for rapid type-specific(Chow et al., 1998) or genus-specific detection (Hafliger et al., 1997; Pfeffer et al., 1997). *In situ* RT-PCR was used to localize the virus in the specimens (Walker et al., 1998; Qureshi et al., 1997). RT-PCR-ELISA (Whitby et al., 1997) was also developed and much more sensitive than southern blot hybridization. RT-PCR coupled with microplate colorimetric assay (Legeay et al., 1997) can be used to quantitate PCR products.

RT-PCR targeting of bacterial ribosomal RNA has been frequently used to detect bacteria. This method has several advantages. One of them is that bacterial rRNA has conserved and variable regions. This makes it convenient to find general as well as specific target sites for PCR primers. Second, various databases of rRNA sequences such as Ribosomal Database Project and Gene Bank are available so that phylogenetic analysis (Rossello-mora et al., 1995; Gillan et al., 1998) and primer design can be performed. Third, each bacterial cell contains 1,000 to 10,000 copies of rRNA, detection of rRNA should impart increased sensitivity over assays based on the detection of a

single copy or even multiple copies of genomic sequences. For example, RT-PCR assay targeting the 16S rRNA of *Mycobacterium leprae* (Kurabachew et al., 1998) or *Treponema pallidum* (Centurion et al., 1997) was used to detect low numbers of viable organisms in samples.

RT-PCR targeting bacterial mRNA has also been used to detect viable bacteria because bacterial mRNA has an extremely short half life, averaging only a few minuets. Previously, the presence of viable *Mycobacterium tuberculosis* (Jou et al., 1997) and *Listeria monocytogenes* (Klein and Juneja, 1997) were detected by using RT-PCR targeting bacterial mRNA.

Efforts have been made to detect protozoans from environments. Procedures used include flow cytometry (Vesey et al., 1993), laser scanning (Anguish and Ghiorse, 1997) and immunomagnetic separation (Campbell and Smith, 1997). The sensitivity of protozoan detection was greatly increased by concentrating cyst and oocysts with filters, selectively capturing mRNA with oligo $(dT)_{25}$ magnetic beads and then performing RT-PCR. It was indicated that low numbers of viable *Giardia* cysts and *Cryptosporidium parvum* oocysts were detected in water samples and the method was more sensitive than using immunofluorescence assay (Kaucner and Stinear, 1998).

MATERIALS AND METHODS

Bacterial Strains

The following bacteria were used in this study: *Salmonella typhimurium* (ATCC 19585), *Escherichia coli* (ATCC 15597), *Klebsiella pneumoniae* (ATCC 13883), *Proteus vulgaris* (ATCC 13315), *Staphylococcus aureus* (ATCC 12600), *Pseudomonas aeroginosa* (ATCC 10145), *Duganella zoogloeoides* (ATCC 25935), '*Z. ramigera*' I-16-M (ATCC 19623), and *Z. ramigera* 106 (ATCC 19544). *Z. ramigera* 106 (ATCC 19544), '*Z. ramigera*' I-16-M (ATCC 19623), and *Duganella zoogloeoides* (ATCC 25935) were grown in YP medium (2.5 g/l yeast extract, 2.5 g/l peptone) for 36-48 hours at 28^{0}C. All other bacteria were grown in Tryptic Soy Broth (Difco Laboratories, Detroit, MI) for 24 hours at 37^{0}C.

Z. ramigera Enriched Biofilm Development

Phenol (Acros Organics, Pittsburgh, PA) was added to 100 ml samples from wastewater treatment plants or surface water contained in 250-ml beakers daily to provide an initial concentration of 50 µg/ml. The samples were incubated at ambient temperature (approximately 25^{0}C) for up to one week. During incubation, the samples were periodically examined for the development of a biofilm that contained finger-like projections characteristic of *Z. ramigera.*

Column Studies

Raw sewage was obtained from University of Florida Water Reclamation Facility (Gainesville, FL). The raw sewage was filtered through cheese cloth (Fisher Scientific, Springfield, NJ) to remove large particles from the water before passing through the column.

Ottawa sand (Fisher Scientific, Springfield, NJ) was packed into an acrylic column (1.5cm ID x 0.5 m). The raw sewage with 50 μg/ml phenols was passed through the column in inflow mode. The treatment lasted for two weeks. The sand was then taken out from the column, washed with deionized water for 3 times and ready for observation under a scanning electron microscope.

Scanning Electron Microscopy (SEM)

Mid-log phase of *Z. ramigera* 106 (ATCC 19544) culture was centrifuged and washed twice with deionized water. The sample was dehydrated by soaking for five minutes serially in increasing ethanol solutions (25%, 50%, 75%, 95% and twice in 100% ethanol). It was then fixed by hexamethyldisilizane (HMDS). The culture was then mounted on a nucleopore filter (Fisher Scientific, Pittsburgh, PA) and sputter-coated with gold particles for 5 minutes. The sample was viewed on the Hitachi S-4000 Field Emission SEM. Natural biofilms that developed on sand filter media after the passage of phenol fortified (50 μg/ml) wastewater were also processed and observed on a SEM as described above.

Effect of Antibiotics on *Z. ramigera* Growth

All antibiotics were purchased from Sigma Chemicals (St. Louis, MO). Stock solutions for trimethoprim (0.5 mg/ml) were made by dissolving 0.05 g of trimethoprim in 5 ml benzyl alcohol and 5 ml deionized water. The solutions were passed through a 0.45μm filter (Fisher Scientific, Pittsburgh, PA). Stock solutions for penicillin (7.5 mg/ml), tetracycline (25 mg/ml), streptomycin (125 mg/ml), sulfadiazine (5 mg/ml), and cephalosporin (100 mg/ml) were made in deionized water and filter sterilized.

Tests were carried out by adding different concentrations of antibiotics into YP liquid medium. *Z. ramigera* (ATCC 19544) was inoculated and incubated at 28^0C for 4-5 days. Growth was determined by comparing growth in tubes without antibiotics to growth in tubes with antibiotics.

Isolation of *Z. ramigera* from Natural Environments

In order to make m-toluic acid isolation media, the following solutions were used. (A). Potassium phosphate solution (0.06M, 100x) was made by adding 1.0 g of K_2HPO_4 to 100 ml deionized water. The solution was adjusted pH to 7.2 and autoclaved for 15 min; (B). M-toluic acid (50 mg/ml) stock solution (1,000x) was made by mixing 5 g of m-toluic acid, 1ml solution A, and 5 ml of 1N NaOH. The mixture was heated until the m-toluic acid was completely dissolved. The pH was adjusted to 7. The solution was passed through a 0.2 μm pore size filter; (C). Salt solution (100x) was made by adding 2 g of $MgSO_4$, 3.75 g of $(NH_4)_2SO_4$ and 0.02 g of $CaCl_2$ to 100 ml deionized water;

(D).Yeast autolysate solution (100X) was made by adding 0.1 g of yeast autolysate into 100 ml deionized water.

To make the isolation medium, 1 ml solution C, 1 ml solution D, 97 ml deionized waters and 1 gram of agar were mixed. After autoclaving and cooling to 55^{0}C, 1 ml of solution A, 0.1 ml solution B and appropriate amount of sulfadiazine and/or trimethoprim were added.

Raw sewage was obtained from University of Florida Water Reclamation Facility (Gainesville, FL). Lake water was obtained from Lake Alice (Gainesville, FL). *Z. ramigera* enriched biofilm developed over raw sewage was conducted by adding 50 μg/ml phenol into raw sewage and incubated at room temperature for 4-5 days. *Z. ramigera* enriched biofilm developed over lake water was conducted by adding 50μg/ml phenol into lake water and incubated at room temperature for 7-10 days. The microorganisms from the biofilms were streaked on the m-toluic acid isolation medium combined with 1μg/ml trimethoprim and/or 10μg/ml sulfadiazine. Hard colones on the agars resembling *Z. ramigera* (Unz and Farrah, 1972) were selected and re-streaked on the same medium 2-3 times for purification.

Mixed liquor suspensions were obtained from University of Florida Water Reclamation Facility (Gainesville, FL). Mixed liquor suspensions were centrifuged at 5,000 rpm for 5 min. The pellet and an equal volume of the supernatant were blended for 1-2 min to release the microorganisms from the floc. The blended activated sludge was streaked on the m-toluic acid isolation media combined with 1 μg/ml trimethoprim

and/or 10 μg/ml sulfadiazine. The isolated bacterial colonies were purified by re-streaking the same medium 2-3 times.

Tests for nitrate reduction, oxidase, catalase, Gram stain, urea hydrolysis, glucose utilization, indole production (Cappuccino and Sherman, 1992), and *meta* cleavage of benzene derivatives (Unz and Farrah, 1972) were conducted to identify the isolated strains.

Isolation of *Z. ramigera* Extracellular Polymer

Z. ramigera 106 was inoculated in YP medium and incubated at 28^{0}C until log-phase. The cultures were centrifuged and washed twice with deionized water, then suspended in equal volume of 0.4 M K_2HPO_4 (final concentration is 0.2 M) and blended (Tekmar, Cincinati, Ohio) for 1 min. The mixture was centrifuged for 10 min at 27,000 x g and the pellet was discarded. Cetyltrimethylammonium bromide (CTAB) was added to the supernatant to a final concentration 0.8% (wt/vol). The solution was centrifuged after 4h at room temperature. The precipitate was mixed with 10 volumes of 0.5 M NaCl and the mixture was centrifuged. The supernatant fraction was dialyzed against deionized water at 4^{0}C for 24 hours. The dialyzed sample was dried and washed with 80% ethanol to remove the residual CTAB (Farrah and Unz, 1976).

Polyclonal Antibodies Production

For production of the antibody against cell walls, log-phase *Z. ramigera* 106 cultures were washed twice and suspended in deionized water. The suspension was

adjusted to pH 10 by using 1N NaOH and boiled for 3 min, then cooled. The pH was readjusted to 10 and the suspension was boiled for another 3 min. Finally, cells were centrifuged at 10,000 rpm for 10 min and washed twice with deionized water. Microscopic examination with India Ink revealed that cells were devoid of the extracellular polymer. Formalin (2%) was used to fix the cells. A fraction of the cells was hydrolyzed in 1N NaOH and a protein assay was performed (Protein Assay Kit, Sigma Chemicals, St. Louis, MO). Cell suspensions were adjusted to 200 mg/ml protein concentrations in PBS buffer and were mixed with an equal volume of Freunds complete adjuvant (Sigma Chemicals, St. Louis, MO). One ml of the suspension was then injected subcutaneously into a rabbit. After 2 weeks, a 1 ml inoculum of the cells in Freunds incomplete adjuvant was intravenously injected and this was repeated two weeks thereafter. Ten days after the final injection, the rabbits were bled and the antiserum was collected. For the production of antibody against *Z. ramigera* extracellular polymer, the extracellular polymer was isolated from the cells as previously described (Farrah and Unz, 1975). The same procedure was followed for antiserum production using the rabbits. Unimmunized rabbit serum (Sigma Chemicals, St. Louis, MO) was used as negative control in all immunological procedures.

Indirect Immunofluorescence Staining

Samples were spread on microscopic slides, air dried and fixed with 50%, 80% and 96% ethanol sequentially for 3 minutes each time. The rabbit antiserum was diluted

with PBS buffer and added to the slides and incubated for 30 minutes at room temperature in a hydrated chamber. The slides were washed with PBS buffer several times. Then FITC-labeled goat anti-rabbit IgG (Sigma) diluted in PBS was added and incubated for 30 minutes. The slides were then washed twice with PBS buffer. A drop of 10 mg/ml DABCO (1,4-diazabicyclo (2,2,2) octane) (Sigma Chemicals, St. Louis, MO) was added to enhance fluorescence and a cover slip was placed on the slide. The slides were examined by phase-contract and epifluorescence microscopy (Farrah and Unz, 1975).

Enzyme Immunostaining

All chemicals and reagents used were obtained from Sigma Chemicals (St. Louis, MO). Samples were spread on microscopic slides, air dried and fixed with 50%, 80% and 96% ethanol sequentially for 3 minutes each time. The rabbit antiserum was added and allowed to react for 30 minutes. The slides were washed with PBS buffer. Then biotin-conjugated anti-rabbit IgG (1/500dilution with PBS buffer) was added and allowed to react for 30 minutes. The slides were washed with PBS buffer and peroxidase-labeled avidin was added (0.64 units of peroxidase /ml) and reacted for 30 minutes. After the slides were washed with PBS buffer, 0.5 ml of the substrate, aminoethyl carbozole (AEC) solution (3 volumes of 4.0 mg/ml AEC dissolved in N, N-dimethylformamide plus 7 volumes of 0.05M acetate buffer [pH 5.0]) was added. Then, 1 µl 30% H_2O_2 was added to the slides to activate the substrate (Cleveland and Richman, 1987). After 10 min, the slides were washed with PBS buffer and checked under a light microscope.

Immunoassay by Scanning Electron Microscopy (SEM)

The scum layers of biofilm were put into microcentrifuge tubes and were washed 3 times by Tris-buffered saline (TBS) (pH 7.4) thoroughly and fixed by 4 % paraformaldehyde (PFA) solution for 30 min on ice or 10 min at room temperature. Samples were washed once by TBS (pH 7.4) and incubated with 1% gelatin in TBS for 10 minutes. Three 5 minutes incubations with 0.02 M glycine in TBS were used to preblock. Then samples were washed by 5 minutes incubation with TBS (with 1% BSA). Goat serum (1:10) was added to block for 10-20 minutes and was washed with TBS once. A rabbit antiserum (1:500) was added and reacted for 1 hour at room temperature. Samples were washed for 5 minutes three times with TBS (with 1% BSA, pH 7.4) followed by two 5 minutes washes with TBS (with 1% BSA, pH 8.2). Gold labeled goat anti-rabbit IgG (1:100) was added and incubated for 1 hour at room temperature. Samples were washed three times for 5 minutes with TBS (pH 7.4) followed by fixation with Trump's solution (4% formaldehyde, 1% glutaraldehyde in phosphate-buffered saline, pH 7.4) for 30 minutes. Samples were washed three times for 5 minutes with TBS (pH 7.4) followed by four 5 minutes ultra pure water washes. Silver enhancement was performed for 5 minutes and was followed by three 5 minutes water washes. Samples were then mounted on nucleopore fiter (Fisher Scientific, Pittsburgh, PA). Dehydration and fixation were performed using gradient alcohol dehydration: 25%, 50%, 75%, 95% for 5 min each, 100% for 5 min twice followed by 5 minutes hexamethyldisilizane (HMDS) washes twice. Samples were dried, carbon coated for 10 seconds, and observed under a Hitachi S-400 Field Emission SEM.

Determination of Most Probable Number (MPN) of *Z. ramigera* in Wastewater Treatment Plants and Lake Water

Autoclaved bottles were prepared prior to sample collection. Raw sewage, mixed liquor suspension, unchlorinated effluent and chlorinated effluent were obtained from the University of Florida Water Reclamation Facility (Gainesville, FL). Ten mg/l sodium thiosulfate was added immediately to collected chlorinated effluent. Mixed liquor suspension, primary aerobic digested sludge and final aerobic digested sludge were obtained from the Kanapaha Water Treatment Plant (Gainesville, FL). Lake water was obtained from Lake Sheelar, Lake Geneva, Lake Johnson (Clay county, FL), Lake Alto, Santa Fe Lake, Lake Alice, and Lake Bivans Arm (Alachua county, FL).

In the University of Florida Water Reclamation Facility, raw sewage is mixed with returned sludge in the mixed liquor tank. After the activated sludge process, sludge solids are settled and separated from the supernatant. The mixed liquor supernatant is then treated by rapid sand filtration. The effluent from the rapid sand filter is unchlorinated effluent. The unchlorinated effluent is then chlorinated in the chlorination basin. Part of the chlorinated effluent goes to Lake Alice. In the Kanapaha Wastewater Treatment Plant, after the activated sludge process (mixed liquor stage), the sludge goes through primary and secondary aerobic digestion.

A three-tube MPN procedure (American Public Health Association, 1989) was used to determine the concentration of *Z. ramigera* and total bacteria in wastewater samples. For determining the MPN for total bacteria, samples were serially diluted in YP medium and incubated for up to 2 weeks at 28^0C. For determining the MPN for *Z.*

ramigera, samples were serially diluted in a filter sterilized raw sewage. The samples were then supplemented with 50μg/ml phenol daily and incubated at 28^{0}C up to 2 weeks. The scum layer that developed was examined microscopically for the presence of typical finger-like projection and/or cells that reacted with *Z. ramigera* antisera as described above.

Chlorophyll concentrations (μg/l) in lakes were determined by pigment extraction with ethanol (Sartory and Grobbelaar, 1984), followed by spectrophotometrical measurement (method 10200H(2c), American Public Health Association, 1989). Total phosphorus concentration (μg/l) in lakes were determined by a persulfate digestion (Menzel and Corwin, 1965), followed by the procedures of Murphy and Riley (1962). Total nitrogen concentrations (μg/l) were determined using the procedure described by Bachmann and Canfield Jr. (1996).

RT-PCR

RNase free water was obtained by treating the water with 0.1% DEPC (diethyl pyrocarbonate) (Sigma Chemicals, St. Louis, MO). Deionized water with 0.1% DEPC was shaken vigorously to bring the DEPC into solution. The solution was then incubated for 12 hours at 37^{0}C and autoclaved for 15 minutes to remove any trace of DEPC. All solutions were made by using RNase free water. Isoporpanol, Mops (3-morpholino) propanesulfonic acid), β-mercaptoethanol, 10 x Tris-EDTA buffer and lysozyme were purchased from Sigma Chemicals (St. Louis, MO). RNase AWAY (Fisher Scientific,

sprinfield, NJ) was used to keep working areas and pipets RNase free. RNase free pipet tips and PCR tubes were obtained by autoclaving overnight.

The sequence of *Z. ramigera* (ATCC 19544)16S rRNA was retrieved from Gene Bank (ZR16SRRNA). The upstream and downstream primers were specific for *Z. ramigera* 106 16S rRNA and purchased from Genosys (The Woodlands, Texas). Sequence for the upstream primer is CCG ATG TCG GAT TAG CTA GTT GG (position 219 to 242). Sequence for the downstream primer is AAT GAG TCT CCT CAC CGA ACA ACT AG (position 813 to 836). The lyophilized primers were suspended in DNase and RNase free water and incubated at 37^{0}C for complete dissolution.

Bacterial 16S rRNA were isolated by using QIAGEN RNA/DNA mini kit (Valencia, CA). Briefly, 5x 10^8 bacterial cells were centrifuged and resuspended in 50 µl of lysozme-containing TE buffer for 5 min. Then the lysing buffer was added to the solution as described in the kit. The 16S rRNA was isolated exactly by using the 'low molecular weight RNA isolation' procedure in the kit. QIAGEN tip is anion-exchange column which can be used to selectively isolate DNA, RNA and low molecular weight rRNA. RT-PCR kit was purchased from Promega (Pittesburgh, PA). RT-PCR was performed in a total volume of 50 µl reaction mixture containing 0.2 mM deoxynucleoside triphosphate, 0.5 mM $MgSO_4$, 5 U of AMV reverse transcriptase, 5 U of *Tfl* DNA polymerase, 0.5 µM of the primers which are specific for *Z. ramigera* 106 16S rRNA, 1 µg of template and 1X reaction buffer. The cycling profile involved 48^{0}C reverse transcription for 45 minutes, 94^{0}C AMV reverse trasriptase inactivation and

RNA/cDNA/primer denaturation, 40 cycles of denaturation at 94^{0}C for 30 seconds, annealing at 60^{0}C for 1 minute and extension at 68^{0}C for 2 minutes, followed by 1 cycle of final extension at 68^{0}C for 7 minutes.

Aliquotes (6μl) of the PCR products were electrophoresed through 2% agrose gel (Sigma Chemicals, St. Louis, MO). Gel star (FMC Bioproducts, Rockland, ME) was used to locate the DNA bands.

Glycosyl Composition Analysis by GC/MS

Z. ramigera extracellular polymer was isolated as described on page 30. After 80% ethanol treatment, deionized water was used to wash ethanol from the extracellular polymer. The extracellular polymer then dispersed into deionized water and homogenized by blending for 1 min. GC-MS analysis of TMS methyl glycosides was used to determine the glycosyl composition of *Z. ramigera* extracellular polymer. The TMS methyl glycosides were prepared by methanolysis in methanolic 1M HCl at 70^{0}C for 16 hours, re-N-acetylated and trimethysilylated (Chaplin, 1986). A Shimadzu QP5000 GC-MS work station was used for GC-MS analysis by staffs at the University of Florida Glycobiology Core Lab. During the experiment, external standards which consisted of known molar concentration of inositol and each monosaccharide in the hydrolysate were used. Calculations of each monosaccharide were based on response factors which was the ratio of peak area for each monosaccharide to a molar of the monosaccharide relative to that for inositol.

Zeta Potential Measurement

Z. ramigera 106 (ATCC 19544), *'Z. ramigera'* I-16 M (ATCC 19623), *Duganella zoogloeoides* (ATCC 25935) were inoculated in YP medium and incubated at 28^0C until log phase. The cultures were centrifuged, rinsed twice with deionized water. The bacteria were resuspended in distilled water (pH 7.0) to a final concentration of 1.0×10^7 CFU/ml. Distilled water was chosen to minimize any change shielding within the invalidation of the assumed equality between the measured zeta potential and the surface potential. The zeta potential measurements were carried out with the use of a Brookhaven Instruments Zetaplus Model V3.21 zeta potential analizer (Holtsviller, NY) (Turesdail et al., 1998).

The Effect of Chlorine on Bacteria in Biofilm

Phenol was added to 100 ml raw sewage in 250-ml beakers daily to provide an initial concentration of 50 μg/ml and incubated at room temperature for 4-5 days. When there were many finger-like projection in the biofilm, different concentrations of chlorine (Clorox Bleach) were added to the beakers for 15 min. Then a HACH test kit (Loveland, Colorado) was used to evaluate free chlorine and total chlorine. The reaction was stopped by adding sodium thiosulfate to a final concentration of 0.2%. INT (0.2 %) (Sigma Chemicals, St. Louis, MO) was added to the beaker to a final concentration of 0.02% and incubated at room temperature in the dark for 30 minutes. Formaldehyde (37%) was added to a final concentration of 0.37% to stop the reaction. The scum layers of biofilms were mounted on clean slides. The slides were air dried and gently fixed by

heat. Next, 0.05 % malachite green was added to the slide and reacted for 1 min. Deionized water was used to wash the slides. The slides were then observed under bright field microscope (Bitton and Koopman, 1982; Dutton et al., 1983).

The Effect of Chlorine on *Z. ramigera* With or Without Extracellular Polymer

Z. ramigera 106 was inoculated in YP medium or low Mg^{++}, Ca^{++} salt minimal medium (5.0 g glycerol, 0.3 g $(NH_4)_2SO_4$, 0.8 g sodium lactate, 1.0 g KNO_3, 0.05 g K_2HPO_4, 0.1 g $CaCl_2$, 0.1 g $MgSO_4$) and incubated at 28^0C until log phase. The cultures were centrifuged at 2,000 rpm for 5 min. The pellet which contained mostly flocs was discarded. The supernatant was further centrifuged at 10,000 rpm for 10 min. The pellet which contained both free cells and cells in flocs was resuspended in deionized water. Different concentrations of chlorine were added into the suspension for 15 min. The determination of chlorine concentration and the MINT test were performed as described above.

Bacteriophage Assay

PRD1 is an icosahedral lipid phage characterized by a diameter of 62nm. The isoelectric point of PRD1 is between 3 to 4 in a calcium-phosphate buffer (10^{-4} M Ca) (Pieper et al., 1997). It grows on its host *Salmonella typhimurium* (ATCC 19585). MS2 is an icosahedral phages with an average diameter of ~25nm and has isoelectric point of 3.9 (Lin et al., 1997). It grows on its host *Escherichia coli* C3000 (ATCC 15597). Both viruses were assayed as plaque- forming unit method (Snustad and Dean, 1971).

Preparation of Aluminum Hydroxide Coated Sand

A U. S. Standard No. 25 sieve was used to collect sand particles of 600-700 μm in diameter from 25 x 30 meshes Ottawa sands (Fisher Scientific, Springfield, NJ). The graded sand was rinsed with deionized water until the supernatant was clear. The sand was then air dried. The sand was placed in 1.0M of $AlCl_3.6H_2O$ (Fisher Scientific, Springfield, NJ) solution for 30 minutes. The excess solution was drained off. Then the sand was air-dried for 24 hours. The dried sand was then socked in 3.0M ammonium hydroxide for 10 minutes to precipitate aluminum hydroxides on the sand. The sand was air dried and then rinsed with deionized water vigorously to remove loose precipitate, then air dried again.

Wastewater Exposure of the Coated Sands

Uncoated sand and a portion of coated sand without exposure to wastewater were used as negative and positive controls, respectively. The coated sand was packed into acrylic columns (3.2 cm I. D. x 1.5 m). Wastewater effluent from University of Florida Water Reclamation Facility was used in this study. The typical compositions of wastewater effluent were: 1.0 mg/l total Kjeldahl nitrogen, 1.2 mg/l orthophosphate, 1.8 mg/l total phosphate, 0.7 NTU turbidity, 795 μmhos/cm conductivity, 8mg/l Mg^{2+}, 73 mg/l Ca^{2+}, 1.2 mg/l NO_3-N, 0.2 mg/l NH_3-N, 44 mg/l $CaCO_3$ (alkalinity), pH 7.2. The experiments were carried out at room temperature. Dechlorination of wastewater effluent was done with sodium thiosulfate. Chlorinated wastewater effluent was obtained by treating water with 10 mg/l ammonia nitrogen followed by titration with sodium

hypochlorite (Clorox Professional Products, Oakland, CA) so that final combined chlorine was 2 mg/l chlorine. The upflow rate was 57 ml/min and thus the superficial velocity was 1.2mm/s. The columns were back washed every 72 hours at a superficial velocity of 17mm/s for 15 minutes to fluidize the sand. Before each sampling, the sand was taken out from the columns and rinsed with filter-sterilized wastewater to remove loosely attached biomass. Portions of the sand were used for a protein assay, zeta potential measurements, and virus removal assay by batch and column experiments. The rest of the sand was returned to the columns and the wastewater passing was continued.

Surface Characterization of the Sands

Protein assays were conducted by the modified Lowry method (Bensadoun and Weinstein, 1976; Peterson, 1977) and the procedure which was described in the Sigma Protein Assay Kit (Sigma Chemical). Briefly, 20 g of the sand samples were immersed in 8.0 ml of 10.0 N NaOH to extract the protein. The supernatant was assayed according to the instruction in the protein assay kit. The zeta potentials of the sands were measured with a streaming potential apparatus as described previously (Chen et al., 1998; Truesdail et al., 1998).

Batch and Column Removal of Bacteriophages

Bacteriophage removals by batch tests were determined as follows: MS2 and PRD1 were diluted in a filter-sterilized (0.2μm) milli-molar ionic strength artificial

groundwater (AGW) (1 L deionized water, 35 mg $MgSO_4.7H_2O$, 12 mg $CaSO_4.2H_2O$, 12 mg $NaHCO_3$, 6 mg NaCl and 2 mg KNO_3) (McCaulou et al., 1994) to produce a final concentration of 10^5 PFU/ml for each bacteriophage. Uncoated sand, aluminum hydroxide coated sand without wastewater exposure, aluminum hydroxide coated sand exposed to dechlorinated wastewater, and aluminum hydroxide coated sand exposed to chlorinated wastewater were used for testing. Briefly, four grams of sand were added to 10 ml of bacteriophage suspension in polypropylene tubes (Fisher Scientific, Springfield, NJ) and the mixtures were shaken for 30 min using a 70 cm diameter wheel which was rotated vertically at 30 rev/min at room temperature. The bacteriophage suspension without sand was also shaken in the wheel along with other samples as a control. All sands were tested in triplicate.

Parallel column tests on days 0, 90 and 110 were carried out as follows: Four acrylic sand columns (1.5 cm I.D. x 1.0 m) were used in upflow mode. The four sand columns were uncoated sands, aluminum hydroxide coated sands without exposure to wastewater effluent, aluminum hydroxide coated sands exposed to chlorinated wastewater, and aluminum hydroxide coated sand exposed to dechlorinated effluent. A suspension containing 10^5 PFU/ml of each bacteriophage in AGW was passed through the column at a rate of 20 ml/min. Effluents representing steady state conditions (71-75 pore volumes) were collected (Chen et al., 1998). Bacteriophage concentrations in inffluent and effluent were analyzed by the plaque assay procedure.

All plaque assays for both batch and column experiments were conducted in triplicates. Virus removal capacities of the sands were indicated in terms of percent removal of MS2 or PRD1. Student's *t* test, with a *p* value of 0.05 was used to define statistical significance.

RESULTS

Morphology of *Z. ramigera*

Z. ramigera is a floc-forming bacteria and produces an extracellular polymer. Previously, negative staining by India Ink indicated that cells are inside the extracellular polymer in both finger-like projections and amorphous flocs (Farrah, 1974). In this study, scanning electron microscopy was used to reveal the detail structure of *Z. ramigera* finger-like projections and amorphous flocs. A section of a branching zoogloeal projection is shown in Fig. 2B. The cells are organized in the finger. A higher magnification view of a zoogloeal projection reveals the presence of fibrils running along and between the cells (Fig. 2A). This fibril is likely the extracellular polymer produced by *Z. ramigera*. The presence of cells within the zoogloeal extracellular polymer is clearly shown in Fig. 2D. In the case of amorphous flocs, cells usually embed inside an excessive extracellular polymer (Fig. 2C).

Previous studies have shown that *Z. ramigera* can utilize several aromatic compounds and these can be used to enrich samples for finger-like zoogloeae (Unz and Farrah, 1972; Williams and Unz, 1983). Examination of sand particles from a column that had received raw sewage supplemented with 50 μg/ml of phenol revealed natural finger-like projection structures that resembled those observed in laboratory cultures of *Z. ramigera* 106 (Fig. 3). The structures are smaller in diameter than those observed in

laboratory cultures (Fig. 2B) but show cells in similar arrangements and with similar fibrils. There were little finger-like projections observed in biofilm developed on the surface of sands which had been passed through wastewater without a phenol supplement (Chen et al, 1998). Therefore, the supplement with phenol is effective for production of *Z. ramigera* enriched biofilm.

Isolation of *Z. ramigera* from Natural Environments

Z. ramigera is sensitive to penicillin, tetracycline, streptomycin and cephalosporin. Trimethoprim-sulfamethoxazole (TMP-SMZ) are used for prevention of opportunistic infection in human immunodeficiency-virus-infected person (Kaplan et al., 1996) and for prevention of relapses in patient with Wegener's granulomatosis in remission after respiratory tract infection (Stegeman et al., 1996). The minimal inhibition concentrations (MIC) of trimethoprim and sulfadiazine for *Z. ramigera* are higher than 1μg/ml and greater then 10 μg/ml, respectively (Table 1). Since a m-toluic acid isolation medium was used for isolation of *Z. ramigera* from natural environments (Unz and Farrah, 1972), m-toluic acid isolation medium and m-toluic acid isolation medium combined with trimethoprim and sulfadiazine was used to isolate *Z. ramigera* from activated sludge, biofilm developed over raw sewage, and biofilm developed from Lake Alice water, respectively. Clones resembled *Z. ramigera* were picked up and further purified and identified. Isolates which were Gram negative, urease positive, catalase positive, oxidase positive, nitrate reduction, *meta* cleavage of catechol, indole negative

and no production of gas or acid from glucose were further identified by indirect immunoassay methods. The isolation efficiency obtained was increased from 64% to over 90% by using the combination media (Table 2). The combination media were especially effective when *Z. ramigera* was not abundant in natural environments, such as in Lake Alice water. No synergistic effect was observed between trimethoprim and sulfadiazine.

Immunological Methods for Detection of *Z. ramigera* from Natural Environments

Initial tests using a FITC-labeled secondary antibody showed that the antibody against *Z. ramigera* 106 cells or the matrix only reacted with *Z. ramigera* 106 but not with other laboratory cultures of the bacteria listed in the Materials and Methods section, including *'Z. ramigera'* I-16-M or *Duganella zoogleoides*. Also, during the course of this investigation, *Z. ramigera* 106 antisera were observed to react with the cells and the extracellular polymer associated with natural finger-like projections but not with other bacteria from natural samples.

A portion of a scum layer that developed over raw sewage enriched with 50 μg/ml of phenol and treated with the primary antibody against the extracellular polymer from *Z. ramigera* 106 followed by FITC-labeled secondary antibody is shown in Fig. 4. A finger-like zoogloeal projection observed using light microscopy and phase contrast optics is shown in Fig. 4A. The same field observed with UV illumination is shown in Fig. 4B. Only finger-like projections characteristic of *Z. ramigera* were observed to fluoresce under UV light.

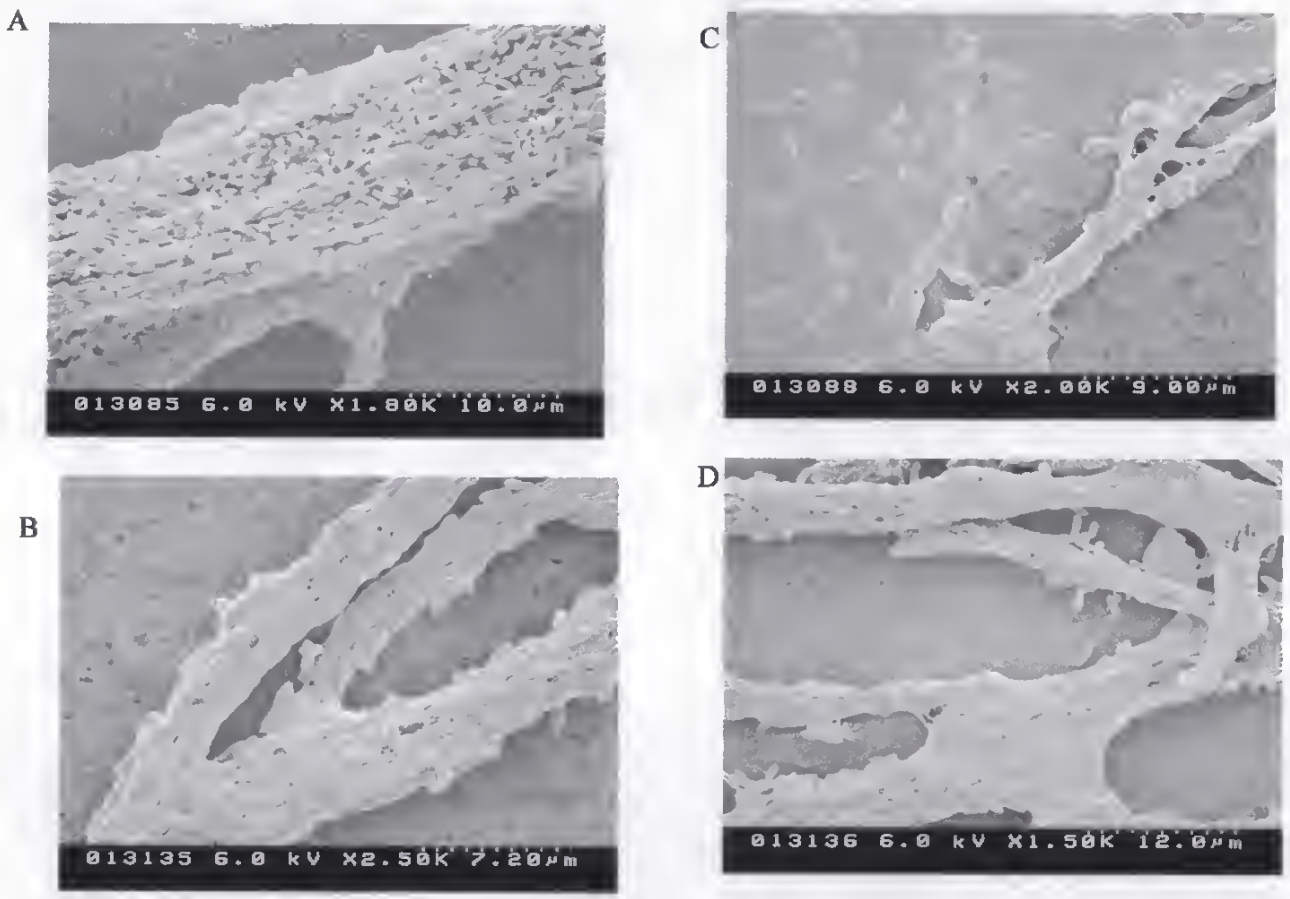

Fig. 2. SEM of *Z. ramigera* 106 (ATCC 19544) which was grown in YP medium (2.5 g/l yeast extract, 2.5g/l peptone) for 48 hours at 28^0C.

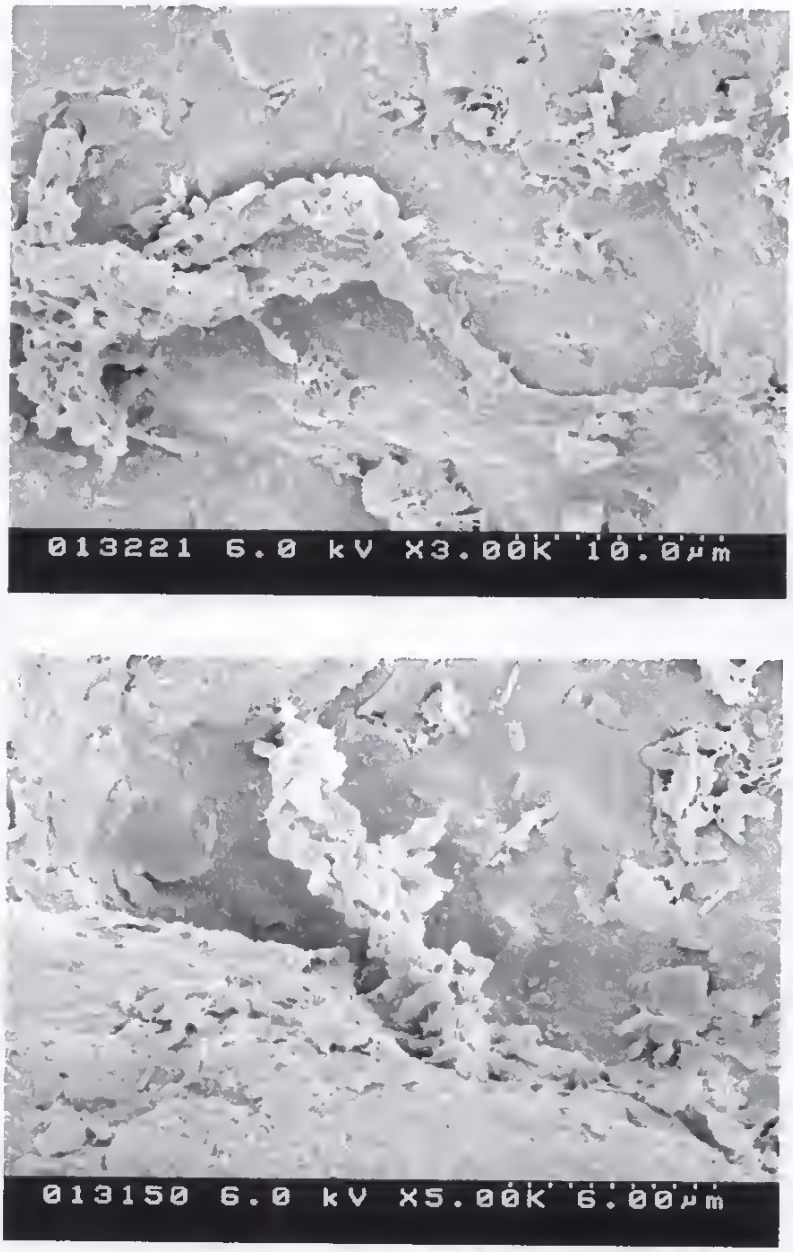

Fig 3. SEM of sand exposed to raw sewage supplemented with phenol for 2 weeks

Table 1. The effect of antibiotics on *Z. ramigera* growth

Antibiotics (μg/ml)		*Z. ramigera* growth
trimethoprim	1	+
	5	-
	10	-
	20	-
	50	-
sulfadiazine	1	+
	5	+
	10	+
	20	-
	50	-

Note: *Z. ramigera* 106 (ATCC 19544) was inoculated in YP medium (2.5 g/l yeast extract, 2.5 g/l peptone) supplemented with different concentrations of trimethoprim or sulfadiazine and incubated at 28^{0}C.

Table 2. The effect of different media on the isolation of *Z. ramigera* from natural environments

Media	No. of samples processed	No. of colonies purified from samples	No. of *Z. ramigera* isolated	Yield (%)
m-toluate	9	14	9	64
m-toluate + 1 μg/ml trimethoprim	9	12	12	100
m-toluate + 10 μg/ml sulfadiazine	9	11	10	91
m-toluate + 1 μg/ml trimethoprim + 10 μg/ml sulfadiazine	9	13	12	92
Total (m-toluate+ antibiotics)	27	36	34	94

Note: Bacteria from activated sludge, biofilms developed over raw sewage and lake waters supplemented with phenol were streaked on m-toluic acid isolation medium as well as the isolation medium combined with 1 μg/ml trimethoprim and/or 10 μg/ml sulfadiazine. Biochemical tests were conducted to identify the isolates.

When the scum layers were examined in a similar manner but using primary antibody against *Z. ramigera* cells followed by FITC-labeled secondary antibody, cells within typical zoogloeal projections fluorescenced while other cells did not. The results were similar to previously published results (Farrah and Unz, 1975).

Observation of the scum layers from samples of raw sewage enriched with phenol and treated with the primary antibody against the cells or extracellular polymer from *Z. ramigera* are shown in Fig. 5. These samples were then treated with biotin-labeled secondary antibody, avidin-peroxidase and substrate AEC and examined using light microscopy. Cells within zoogloeal projections (Fig. 5A) or extracellular polymer surrounding zoogloeal projections (Fig. 5B) were stained dark red by this procedure and are shown as the darker cells and projections in Fig. 5.

SEM images of the scum layers treated with the primary antibody against the *Z. ramigera* extracellular polymer, and gold-labeled secondary antibody are shown in Fig. 6. The label is surrounding the zoogloeal projection in the samples which were examined by using secondary electrons (Fig. 6A, Fig. 6C), and back scattered electrons (Fig. 6B, Fig. 6D) which are produced by the gold particles attached to the secondary antibody. In Fig. 6A and Fig. 6B, it appears that more antibodies had reacted with the end of zoogloeal projection than with the main body of the projection.

The results obtained using similar procedures but with the primary antibody against *Z. ramigera* 106 cells are shown in Fig. 7. The associations of gold particles with

Z. ramigera cells but not with other bacterial cells are shown using images created with secondary (Fig. 7A, Fig. 7C) and back scattered electrons (Fig. 7B, Fig. 7D). No reactions were observed with any of the immunological procedures when unimmunized rabbit serum was used.

RT-PCR

RT-PCR was also used to detect the presence of *Z. ramigera* in samples by using the primers specific for *Z. ramigera* 106 16S rRNA. The region between upstream primer and downstream included the fragment complimentary to the 16S rRNA oligonucleotide probe which was used for *Z. ramigera* detection previously (Rossello-more et al., 1995). When 16S rRNA isolated from *Z. ramigera* 106 (ATCC 19544) was used as a template for RT-PCR reaction, there was 619 bases DNA fragment produced (Fig. 8, lane A). There was no corresponding PCR product when 16S rRNA isolated from *Duganella zoogloeoides* or '*Z. ramigera*' 1-16-M was used in the RT-PCR reaction (Fig. 8, Lane B, Lane C). However, there was the RT-PCR product (619 bases) when 16S rRNA isolated from one of *Z. ramigera* isolates which was isolated from raw sewage by m-toluic acid isolation medium combined with trimethoprim and sulfadiazine (Fig 8, Lane D), or from biofilm developed over raw sewage supplemented with phenol (Fig. 8, Lane E). Therefore, the RT-PCR procedure is sensitive for *Z. ramigera* detection.

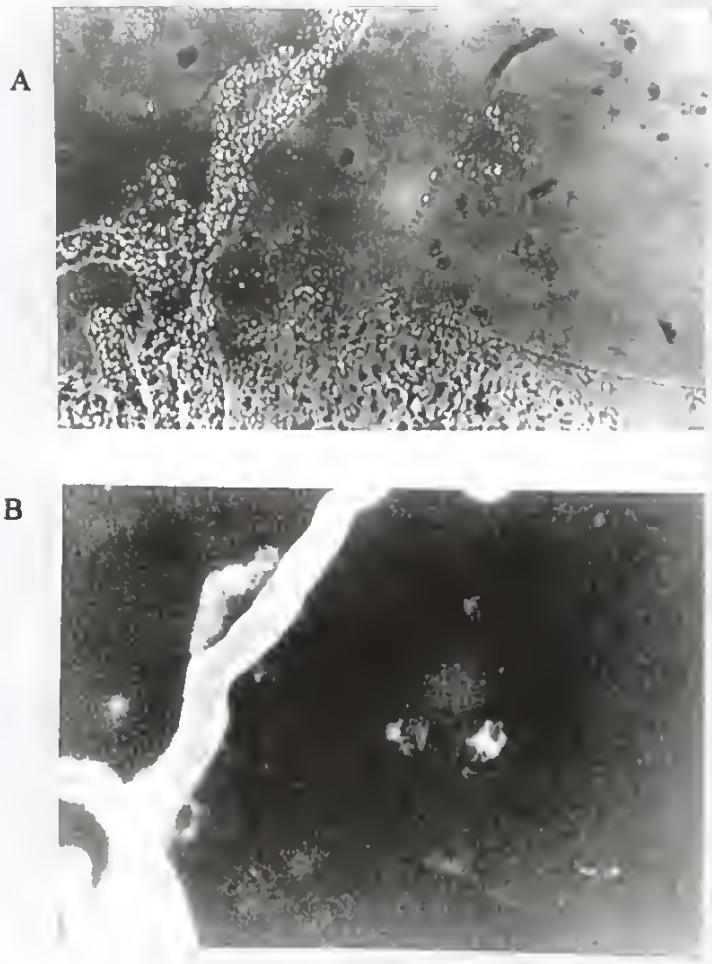

Fig. 4. Indirect immunofluorescence staining of biofilm that developed over raw sewage supplemented with phenol. Samples were treated with rabit antiserum against *Z. ramigera* extracellular polymer with FITC-labeled goat anti-rabbit IgG.

A. phase contrast microscopy B. epifluorescein microscopy

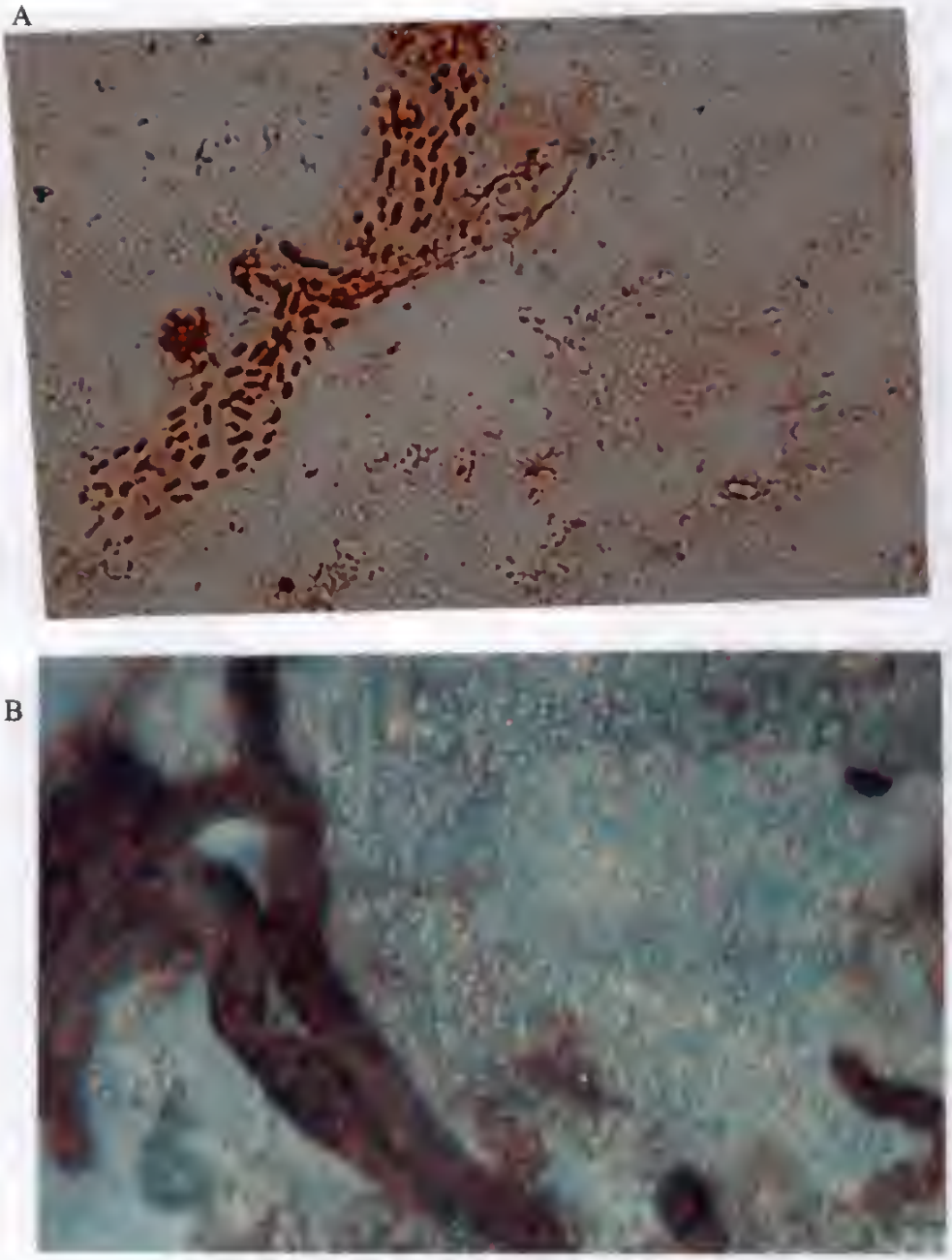

Fig 5. Immunostaining of biofilm that developed over raw sewage supplemented with phenol. Samples were treated with rabbit antiserum for *Z. ramigera* cells (A) or extracellular polymer (B) followed by treatment with biotin-labeled goat anti-rabbit IgG, peroxidase labeled avidin and substrate AEC.

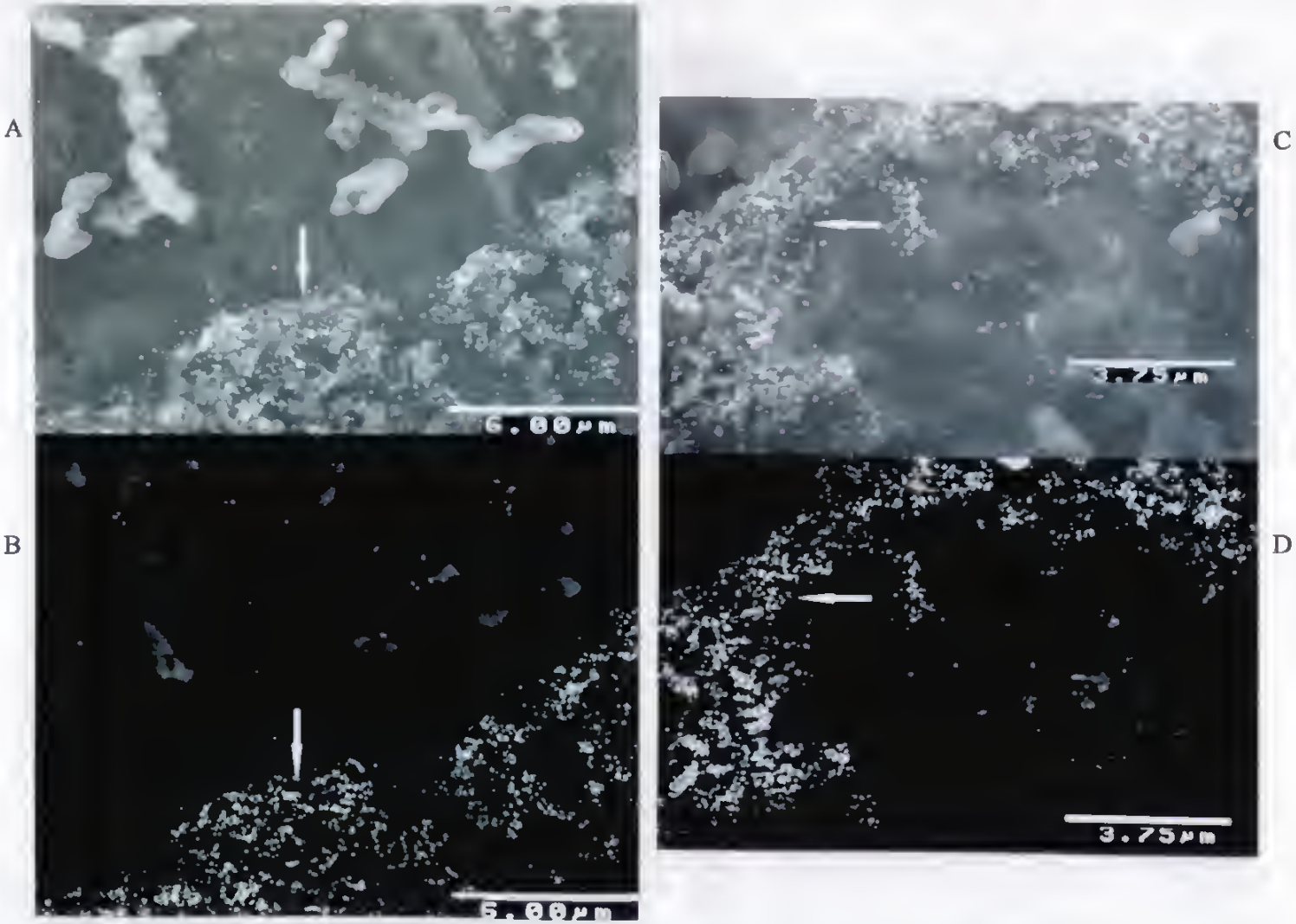

Fig 7. Secondary (A,C) and back scattered (B, D) electron images of SEM photographs of biofilm developed over raw sewage supplemented with phenol Samples were treated with rabbit antiserum for *Z. ramigera* cells followed by treatment with gold-labeled goat anti-rabbit serum.

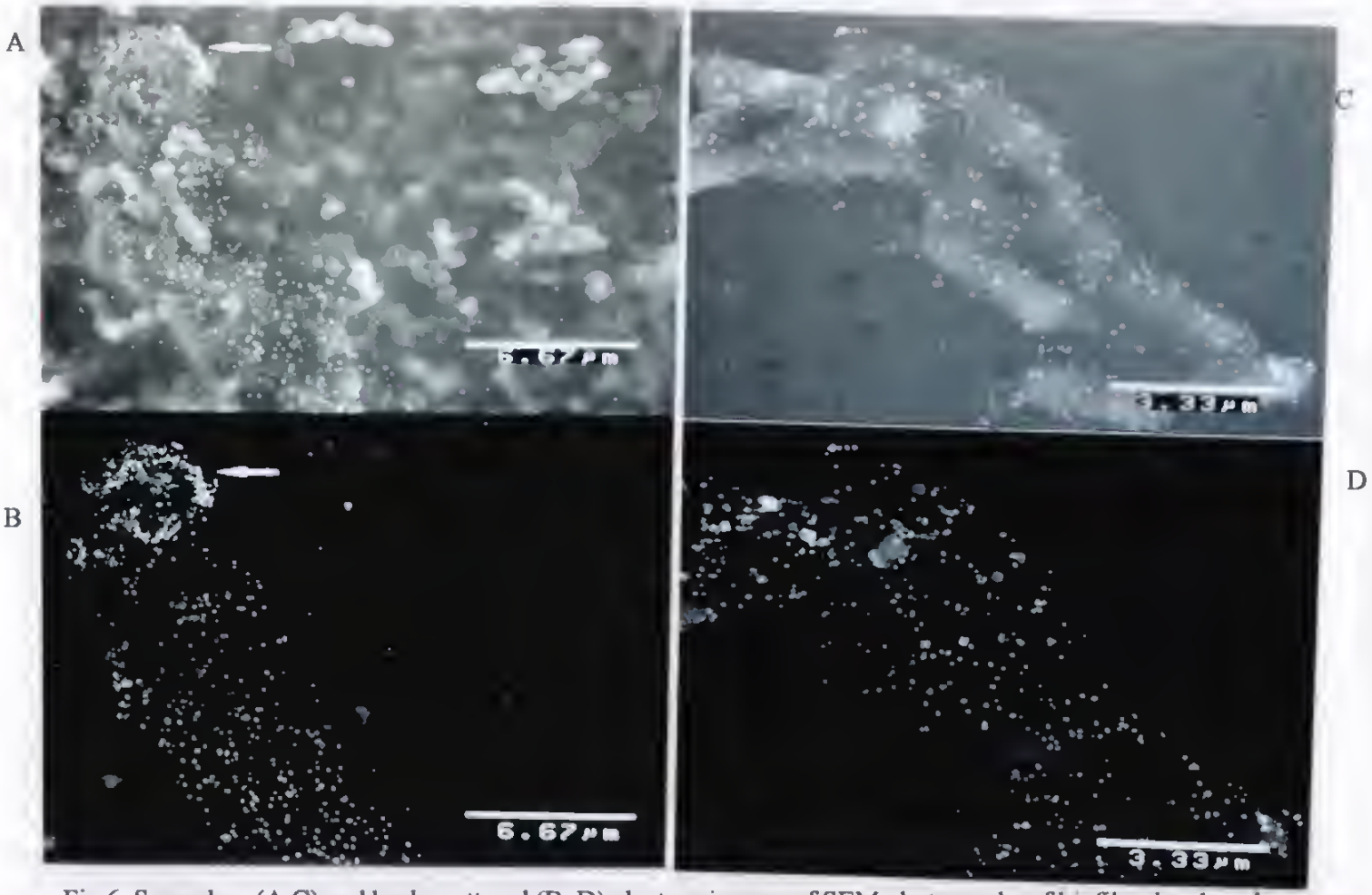

Fig 6. Secondary (A,C) and back scattered (B, D) electron images of SEM photographs of biofilm developed over raw sewage supplemented with phenol Samples were treated with rabbit antiserum for *Z. ramigera* extracellular polymer followed by treatment with gold-labeled goat anti-rabbit serum.

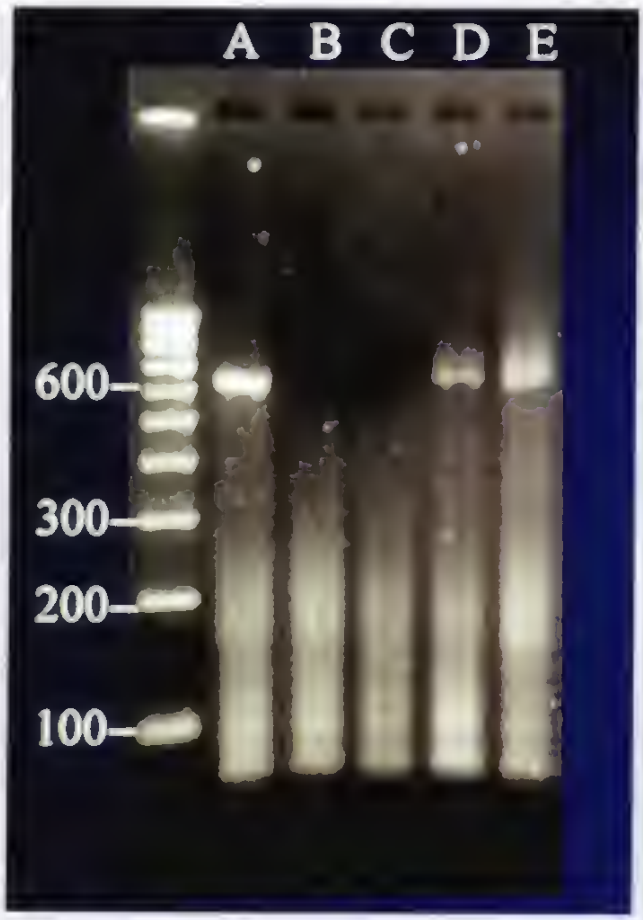

Fig. 8. Electrophoresis of RT-PCR by using primers specific for *Z. ramigera* 106 16S rRNA.

A. 16S rRNA from *Z. ramigera* 106 (ATCC19544)
B. 16S rRNA from '*Z. ramigera*' I-16-M (ATCC19623)
C. 16S rRNA from *Duganella zoogloeoides* (ATCC25935)
D. 16S rRNA from *Z. ramigera* isolated from raw sewage
E. 16S rRNA from biofilm developed in raw sewage supplemented with 50 μg/ml phenol

Distribution of *Z. ramigera* in Wastewater Treatment Plants and in Lakes

Enrichment procedures showed that the finger-like zoogloeal projections which were characteristic of *Z. ramigera* and reacted with *Z. ramigera* 106 antisera were observed in scum layers that developed over samples from all stages of two sewage treatment plants (Tables 3 and 4). The highest percentage of *Z. ramigera* was found associated with samples from the aeration tanks (mixed liquor suspended solids or the supernatant fraction).

In the University of Florida Water Reclamation Facility, the MPN of *Z. ramigera* increased from raw sewage to the mixed liquor suspension, then rapidly decreased from mixed liquor suspension to unchlorinated effluent (Table 3). It was likely that *Z. ramigera* was settled with activated-sludge solids due to its floc-forming characteristic. During chlorination, total bacteria rapidly decreased (from $1.1\pm0.6 \times 10^5$/ml to $2.4\pm1.6 \times 10^1$/ml), while there is little change for the number of *Z. ramigera* between unchlorinated and chlorinated effluent. Thus, the percentage of *Z. ramigera* increased after chlorination (Table 3). Therefore, *Z. ramigera* was more resistant to chlorine than other bacteria in the water. Compared with the chlorinated effluent, the percentage of *Z. ramigera* in Lake Alice was decreased while the total bacteria increased (Table 3). This suggested that there were some nutrients available for bacteria prolification in Lake Alice.

In the Kanapaha Water Reclamation Facility, the percentage of *Z. ramigera* decreased from mixed liquor suspension to primary and final aerobic digested sludge in

both liquid phase and solid phase (Table 4). Overall, *Z. ramigera* could be found in all stages of wastewater treatment processes. The highest concentration of *Z. ramigera* was found in the mixed liquor stage in both wastewater treatment plants (Table 3, Table 4).

According to the content of chlorophyll, a lake is classified as oligotrophic, mesotrophic and eutrophic lake. Distribution of *Z. ramigera* in different types of lakes was also investigated (Table 5). *Z. ramigera* was found in all eutrophic (Lake Alice, Lake Bivans Arm), and mesotrophic (Lake Alto, Lake Santa Fe) lakes tested. However, *Z. ramigera* was present in some of oligotrophic lakes (Lake Geneva, Lake Johnson) , but absent in Lake Sheelar which is also an oligotrophic lake. Low content of nutrients such as total nitrogen and total phosphorous might be the reason for absence of *Z. ramigera* in Lake Sheelar.

Characterization of *Z. ramigera* Extracellular Polymer

GC/MS analysis of an acid hydrolyzed *Z. ramigera* extracellular polymer identified the following components: 1.5% arabinose, 1.38% rhamnose, 0.43% xylose, 4.65% mannose, 0.36% galactose, 1.6% galacturonic acid, 2.7% glucose and 37.7% galactosamine, and another unknown amino sugar (Table 6). This unknown amino sugar is not glucosamine or mannosamine. Due to lack of other standard amino sugars, we could not identify this amino sugar. Total identified sugar in the extracellular polymer was 50%.

Previous work also found two types of aminosugars (glucosamine and possible fucosamine) in *Z. ramigera* extracellular polymer. The ratio between glucosamine and fucosamine was 1:1.5 to 1:2 (Farrah, 1974; Tezuka, 1973). According to this ratio, fucosamine might be almost 50% since 37.7% galactosamine was found in this study. In addition, it was reported that no protein or ether-soluble materials were detected after acid hydrolysis of extracellular polymer (Farrah and Unz, 1976). Therefore, almost 100% of the *Z. ramigera* extracellular polymer is carbobydrate.

It was claimed previously that there was glucosamine in *Z. ramigera* extracellular polymer by paper chromatography. $R_{glucosamine}$ values for galactosamine and glucosamine are similar since galactosamine is a C4 epimer of glucosamine. Therefore, it was hard to differentiate these two sugars by paper chromatography. However, GC/MS analysis indicated the presence of glactosamine instead of glucosamine in the extracellular polymer.

The presence of the extracellular polymer also influences overall surface charge of the cells. Zeta potential bas been used to evaluate overall surface charge of the cells (Truesdail, et al., 1998). Most bacteria are negatively charged due to the predominance of the anionic groups present on the cell surfaces (carboxyl, pbosphate groups). At pH7, overall surface charges of *Z. ramigera* 106 as well as '*Z. ramigera*' I-16-M, *Duganella zoogloeoides*, *E. coli*, *S. typhimuriium*, *S. aureus*, *S. faecalis* were negative (between-17 mv to -40 mv) (Table 7). Therefore, it was suggested that amino groups of aminosugars in *Z. ramigera* extracellular polymer might be acetylated.

Table 3. The distribution of *Z. ramigera* and total aerobic bacteria in the University of Florida Water Reclamation Facility and in Lake Alice

Sources	Total Aerobic Bacteria (MPN)	*Zoogloea ramigera* (MPN)	Percent *Zoogloea ramigera*
Raw sewage	$5.4\pm2.3 \times 10^{6}$/mL	$1.1\pm0.3 \times 10^{4}$/mL	0.20
Mixed liquor supernatant	$7.1\pm2.0 \times 10^{5}$/mL	$4.2\pm2.4 \times 10^{4}$/mL	5.9
Mixed liquor solids	$1.3\pm1.2 \times 10^{9}$/g	$2.0\pm1.6 \times 10^{7}$/g	1.5
Unchlorinated effluent	$1.1\pm0.6 \times 10^{5}$/mL	$8.4\pm5.9 \times 10^{-3}$/mL	7.6×10^{-6}
Chlorinated effluent	$2.4\pm1.6 \times 10^{1}$/mL	$8.0\pm5.7 \times 10^{-3}$/mL	3.3×10^{-2}
Lake Alice	$6.0\pm1.8 \times 10^{3}$/mL	$3.6\pm0.0 \times 10^{-1}$/mL	6.0×10^{-3}

Note: Dilutions of the samples supplemented with 50 μg/ml of phenol were incubated at room temperature for up to 2 weeks. The scum layer that developed was examined for the presence of bacteria within typical finger-like zoogloeae. The samples were also examined using the immunological procedures described in the text to confirm the presence of *Z. ramigera.*

Table 4. The distribution of *Z. ramigera* and total aerobic bacteria in the Kanapaha Water Reclamation Facility

Sources	Total Aerobic Bacteria (MPN)	*Zoogloea ramigera* (MPN)	Percent *Zoogloea ramigera*
Mixed liquor supernatant	$9.7\pm6.9 \times 10^5$/mL	$5.0\pm3.3 \times 10^3$/mL	0.52
First stage aerobic digested sludge supernatant	$3.0\pm1.2 \times 10^7$/mL	$2.5\pm1.4 \times 10^4$/mL	0.08
Second stage aerobic digested sludge supernatant	$3.0\pm1.2 \times 10^7$/mL	$1.2\pm0.9 \times 10^4$/mL	0.04
Mixed liquor solids	$1.2\pm0.5 \times 10^8$/g	$2.0\pm0.4 \times 10^5$/g	0.17
First stage aerobic digested sludge solids	$2.2\pm1.0 \times 10^8$/g	$1.8\pm0.4 \times 10^5$/g	0.08
Second stage aerobic digested sludge solids	$2.3\pm1.4 \times 10^8$/g	$1.4\pm0.2 \times 10^5$/g	0.06

Note: Dilutions of the samples supplemented with 50 mg/L of phenol were incubated at room temperature for up to 2 weeks. The scum layer that developed was examined for the presence of bacteria within typical finger-like zoogloeae. The samples were also examined using the immunological procedures described in the text to confirm the presence of *Z. ramigera*.

Table 5. The distribution of *Z. ramigera* and total aerobic bacterial in different lakes

Classification	Lake	CHL[a] (µg/l)	TP[b] (µg/l)	TN[c] (µg/l)	Total Aerobic Bacteria (MPN/ml)	*Z. ramigera* (MPN/ml)	Percent *Z. ramigera*
Oligotrophic	Sheelar	1.5	2.5	80.0	1.2×10^3	<0.0013	$<1.1 \times 10^{-4}$
	Geneva	1.2	7.7	206.7	5.5×10^2	0.09	0.017
	Johnson	3.0	13.0	230.0	4.8×10^3	0.5	0.009
Mesotrophic	Alto	9.3	11.7	430.0	2.2×10^3	0.2	0.011
	Santa Fe	14.3	14.3	500.0	1.9×10^4	3.6	0.019
Eutrophic	Alice	15.8	327	633.3	6.0×10^3	0.4	0.006
	Bivans Arm	49.7	118.3	1240.0	2.5×10^4	2.4	0.010

Note: Dilutions of the samples supplemented with 50 µg/ml of phenol were incubated at room temperature for up to 2 weeks. The scum layer that developed was examined for the presence of bacteria within typical finger-like zoogloeae. The samples were also examined using the immunological procedures described in the text to confirm the presence of *Z. ramigera*.

a. Total chlorophyll
b. Total phosphorus
c. Total nitrogen

Table 6. Composition of *Z. ramigera* extracellular polymer by GC/MS analysis

Monosaccharides	% (mg/total mg)
Arabinose	1.5
Rhamnose	1.38
Xylose	0.43
Mannose	4.65
Galactose	0.36
Galacturonic acid	1.6
Glucose	2.7
Galactosamine	37.73
Unknown sugar type I[a]	?
Total known sugar	50.35

Note: Acid hydrolyzed extracellular polymer of *Z. ramigera* was re-N-acetylated and trimethysilylated before GC/MS analysis.

a. Unknown Sugar type I is an amino sugar but not glucosamine or mannosamine

Table 7. Zeta potential of bacteria at pH 7

Bacteria	Zeta potential (mv)
Z. ramigera 106 (ATCC 19544)	-24.7 ± 1.93
'*Z. ramigera*' I-16-M (ATCC 19623)	-41.0 ± 0.78
Duganella zoogloeoides (ATCC 25935)	-30.1 ± 1.86
E. coli[a]	-36.5 ± 2.00
S. typhimurium[a]	-17.0 ± 1.00
S. aureus[a]	-36.0 ± 1.00
S. faecalis[a]	-38.5 ± 1.50

Note: The zeta potential of stationary phase bacteria was determined using a Lazer Zee zeta meter.

a. Data from Truesdail et al., 1998

The Effect of Chlorine on Bacteria in Biofilm as well as on *Z. ramigera*

In the MINT test, INT acts as a hydrogen-acceptor. Respiring bacteria will accumulate water-insoluble red INT-formazan crystals through the action of bacterial electron transport system activity. Counter staining with malachite green results in viable bacteria with red color and inactive bacteria with green color. Since the *Z. ramigera* finger-like projection structure is easily distinguished from other bacteria, the bacteria within finger-like projection structures in biofilms were considered to be *Z. ramigera*. The measured values for total chlorine and free chlorine were similar since sodium hypochlorite was used in this study. When a low chlorine concentration (1.0 mg/l) was used, about 80% of the bacteria within finger-like projections were still alive but only 30% of the other bacteria in the biofilm were respiring. At a chlorine concentration of 2.5 mg/l, 65% of the bacteria within finger-like projections were still respiring while most of the other bacteria were inactive. Higher concentration of chlorine (3.5 mg/l) killed all bacteria (Table 8, Fig 9). Therefore, bacteria within finger-like projections can resist higher concentration of chlorine better than other biofilm bacteria.

In order to investigate the influence of the extracellular polymer on *Z. ramigera* inactivation by chlorine, the survival of *Z. ramigera* cells with and without the extracellular polymer were compared during chlorine treatment (Table 9, Fig 10). About 65% of *Z. ramigera* cells with the extracellular polymer were still alive after exposure to 2.5 mg/l chlorine treatments while less than 5% of *Z. ramigera* cells without the extracellular polymer were still alive. Therefore, the *Z. ramigera* extracellular polymer seemed to protect the cells from chlorine inactivation.

Table 8. Effect of chlorine on the respiring activity of bacteria in biofilms

Chlorine concentration (mg/l)	% survival of *Z. ramigera* finger-like projections	% survival of other bacteria
0	99 ± 1	99 ± 1
1.0	80 ± 5	30 ± 2
2.5	65 ± 8	<5
3.5	<5	0

Note: The biofilm that developed over raw sewage supplemented with phenol was treated with indicated concentrations of chlorine for 15 min. The residual chlorine was neutralized with sodium thiosulfate and the MINT test was then performed to detect respiring bacteria.

Table 9. Influence of extracellular polymer on *Z. ramigera* inactivation by chlorine

Chlorine concentration (mg/l)	% survival of cells with extracellular polymer	% survival of cells without extracellular polymer
0	99 ± 1	99 ± 1
2.5	65 ± 4	<5
3.0	<5	0

Note: *Z. ramigera* cells with and without visible extracellular polymer were treated with the indicated concentrations of chlorine for 15 min. The residual chlorine was neutralized with sodium thiosulfate and the MINT test was then performed to detect respiring bacteria.

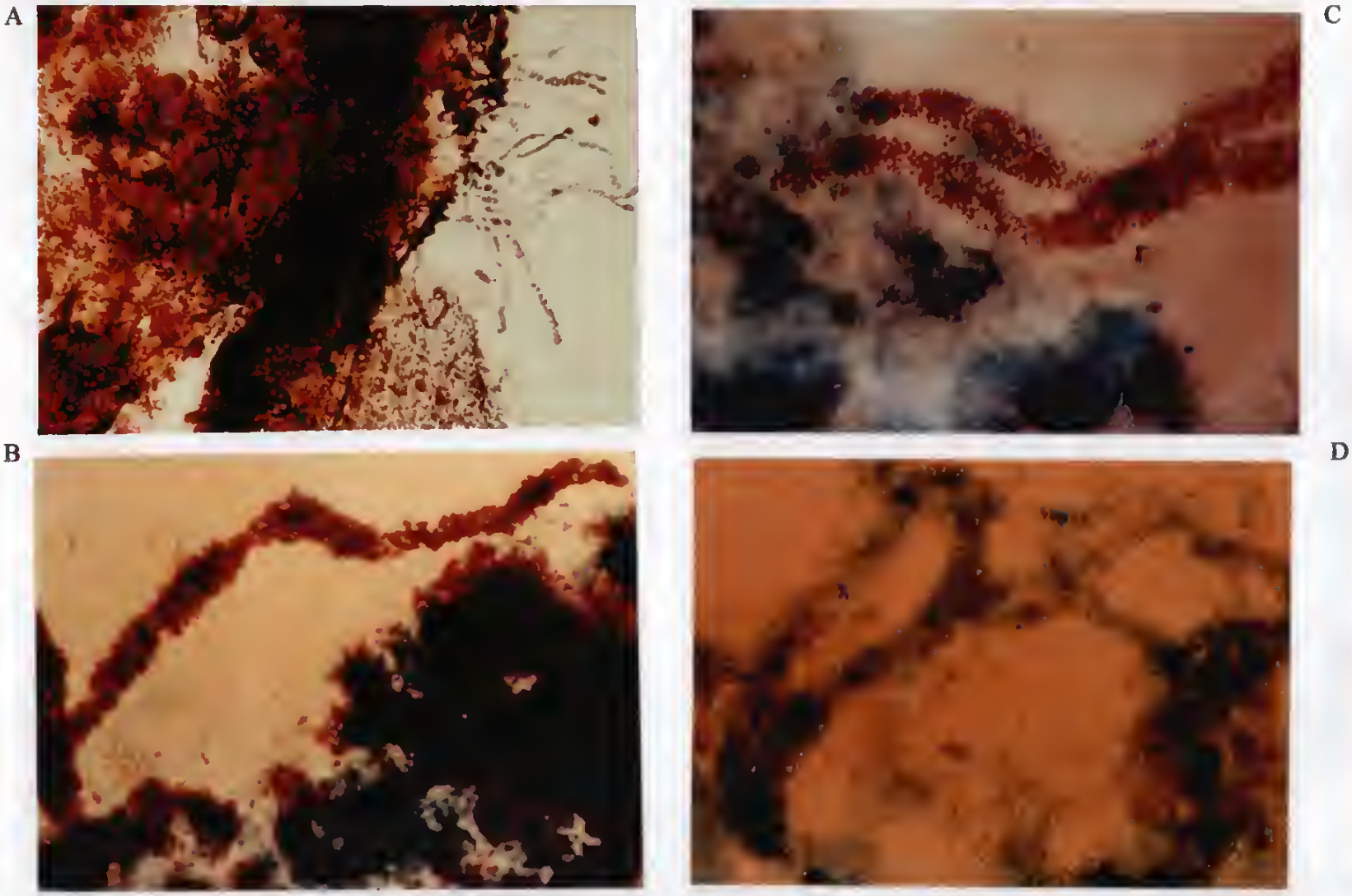

Fig. 9. Effect of chlorine on respiratory activities of bacteria in biofilm
A. 0 mg/l chlorine B. 1.0 mg/l chlorine C. 2.5 mg/l chlorine D. 3.5 mg/l chlorine

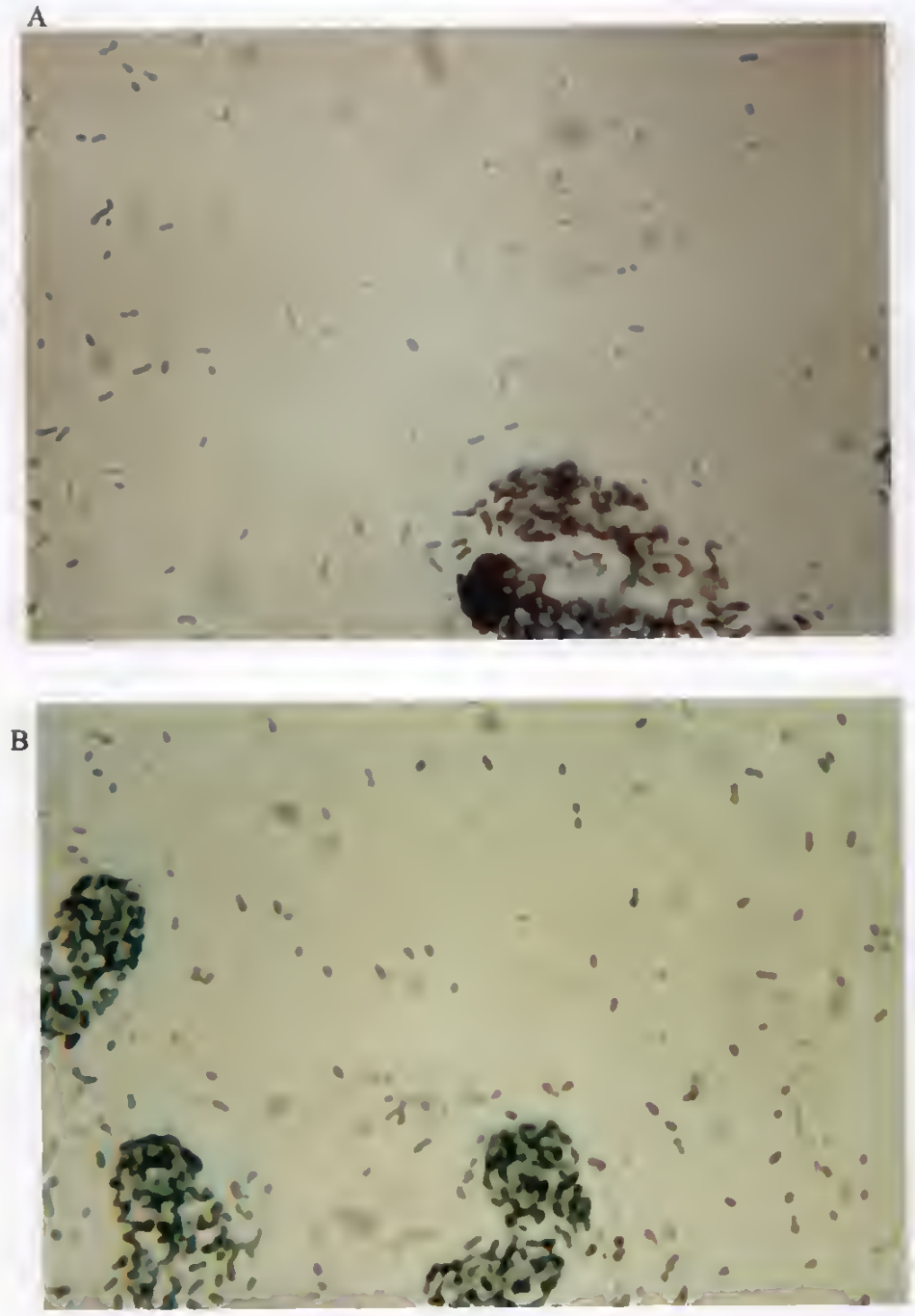

Fig. 10. Influence of the extracellular polymer on *Z. ramigera* inactivation by chlorine.

A. 2.5 mg/l chlorine B. 3.0 mg/l chlorine

Effect of Wastewater on Virus Removal by Aluminum Hydroxide Coated Sands

Characterization of Aluminum Hydroxide Coated Sand Exposed to Either Chlorinated or Dechlorinated Wastewater

Coating by *in situ* precipitation of metallic hydroxides on particles increased the concentration of metals associated with sand particles, their zeta potential and their capacity for removal of microorganisms from water. After coating the Ottawa sands with aluminum hydroxides, the zeta potential of the sand increased from -99mv to +20mv at pH 7 (Table 10). Coating increased the aluminum content of the sand from approximately 0.05 mg/g to 0.4 mg/g (Chen et al., 1998). The coated sands removed 99% of the MS2 and 90% of the PRD1 from water in batch tests (Fig.11 and Fig. 12). The corresponding values for MS2 or PRD1 removal by untreated sand are within 20%.

The zeta potential of aluminum hydroxide coated sands dropped after 1 day exposure to either chlorinated or dechlorinated wastewater (Table 10). At the initial two weeks exposure, the zeta potential of the coated sand exposed to chlorinated wastewater decreased in similar degree as that of the sand exposed to dechlorinated wastewater (from +20mv to 60-80mv). However, after 2 months treatment, the zeta potential of the coated sand exposed to chlorinated wastewater remained around -75mv while the zeta potential of the coated exposed to dechlorinated wastewater was about -40mv (Table 10).

The amount of aluminum on the surface of the sand dropped from 0.4 mg/g to 0.3 mg/g after two weeks of wastewater exposure, then remained approximately constant throughout the experiment (Chen et al., 1998). It was still about 6 fold higher than that

of the uncoated sand (0.005 mg/g). This drop might be due to attrition and/or leaching effects. In addition, since the zeta potential of the sand with 0.4 mg/g aluminum coats was +20mv, zeta potential of the sand with 0.3 mg/g aluminum coats was much less likely about -70mv without other influences. Therefore, the decrease of zeta potential after exposure of the coated sand to wastewater was not likely due to the loss of aluminum content.

Protein assay (Table 11) and SEM (not shown) indicated that biofilm developed on the surface of coated sand which was exposed to dechlorinated wastewater. However, there was no significant biofilm development when chlorinated wastewater was used. Therefore, the effect of biofilm on virus removal by aluminum hydroxide coated sand could be determined by comparing the performance of the sand exposed to dechlorinated wastewater effluent with that exposed to chlorinated wastewater effluent.

Since wastewater effluent used in this study was obtained only after activated sludge and rapid sand filtration processes, there were many organic or inorganic materials in the water. These materials are mostly negatively charged at pH 7 (Sobsey et al., 1984). Evidently, these materials interacted with the aluminum hydroxide coated sand during exposure to chlorinated or dechlorinated wastewater and caused the overall surface charge of the sands decrease (Table 10). It was suggested that organic and inorganic materials in the water 'blocked' the positively charged sites on the sand so that the zeta potential of the sands were drastically decreased (-70 mv) at the earlier stages of the treatment. The amount of biofilm bacteria on the surface of the sand exposed to dechlorinated wastewater was sufficient to maintain the zeta potential of the

sand around -40 mv after 2 months treatment (Table 10 and 11) because the zeta potential of most bacteria are about -20 mv to -40 mv at pH 7 (Truesdail et al., 1998).

In summary, the surface properties of aluminum hydroxide coated sand considerably changed after exposure to dechlorinated or chlorinated wastewater effluent.

Batch Removal of MS2 and PRD1

The aluminum coated sand without wastewater exposure removed about 99.99% MS2 and 90% PRD1. After 1 day exposure to either chlorinated or dechlorinated wastewater, the coated sand were still able to remove about 99.9% MS2 and 65% PRD1 (Fig.11, Fig. 12). The ability of the coated sand for removal of MS2 and PRD1 declined by approximately two thirds after two weeks exposure. After 3 months of treatment, MS2 and PRD1 removal by the sand were not statistically different from that by uncoated sand ($p>0.05$). The performance of the coated sand exposed to dechlorinated wastewater and the sand exposed to chlorinated wastewater were similar for both MS2 and PRD1 removal.

The isoelectric points for MS2 and PRD1 are both between 3 to 4. They were negatively charged in artificial ground water. However, PRD1 is more hydrophobic than MS2 due to the presence of lipid in its protein coat (Kinoshita et al., 1993). The magnitude difference between MS2 (about 99.99%) and PRD1 (about 90%) removed by the coated sand might due to the fact that MS2 is less hydrophobic that PRD1.

Column removal of MS2 and PRD1

In column experiments, the coated sand without wastewater exposure removed 99.9% MS2 and 95.85% PRD1 (Table 12). After long-term exposure of the coated sand to either chlorinated or dechlorinated wastewater, the removal of MS2 and PRD1 was significantly decreased (Table 12). Chlorinated or dechlorinated wastewater exposure resulted in decrease of MS2 and PRD1 removal by the coated sand in similar degree. MS2 and PRD1 rmoval by the coated sand exposed to wastewater for 3 months became statistically similar to the removal by the uncoated sand (p>0.05). The column experiments confirmed the detrimental effect of wastewater exposure for MS2 and PRD1 removal by the coated sand.

Table 10. Zeta potential (mv) of the aluminum hydroxide coated sand after exposure to wastewater

Sands \ Operation Days	0	1	13	60	90	110
Al-coated sand exposed to chlorinated wastewater	+20	-69	-84	-75.5	-79	-75
Al-coated sand exposed to dechlorinated wastewater	+20	-56.6	-76	-43	-40	-36

Note: Sand was washed twice with deionized water. Then the zeta potential was measured as previously described (Chen et al., 1998). The zeta potential of uncoated sand is -99 mv.

Table 11. Protein content (mg/g sand) of the aluminum hydroxide coated sand after exposure to wastewater

Sands \ Operation Days	0	1	13	60	90	110
Al-coated sand exposed to chlorinated wastewater	5.6	7.3	3.8	4.7	9.3	10.3
Al-coated sand exposed to dechlorinated wastewater	5.6	8.3	12.0	57.4	111.0	118.5

Note: Sand was washed twice with deionized water and then mixed with 10N NaOH to extract protein. The sand was allowed to settle and the protein in the supernatant was determined.

Table 12. MS2 and PRD1 removal by sand columns

Virus	Days	Percent Removal by		
		Uncoated sand	Al-coated sand exposed to dechlorinated wastewater	Al-coated sand exposed to chlorinated wastewater
MS2	0	40.4	99.9	99.9
	90	39.1	51.1	63.1
	110	25.23	33.1	33.3
PRD1	0	8.5	95.85	95.85
	90	10.52	40.89	23.28
	110	11.86	23.28	12.15

Note: Sand was packed into columns (1.5 cm I.D. x 1.0m). AGW seeded with MS2 and PRD1 was passed through these columns in upflow mode. At steady state, the MS2 and PRD1 in the column ifluent and effluent samples were assayed.

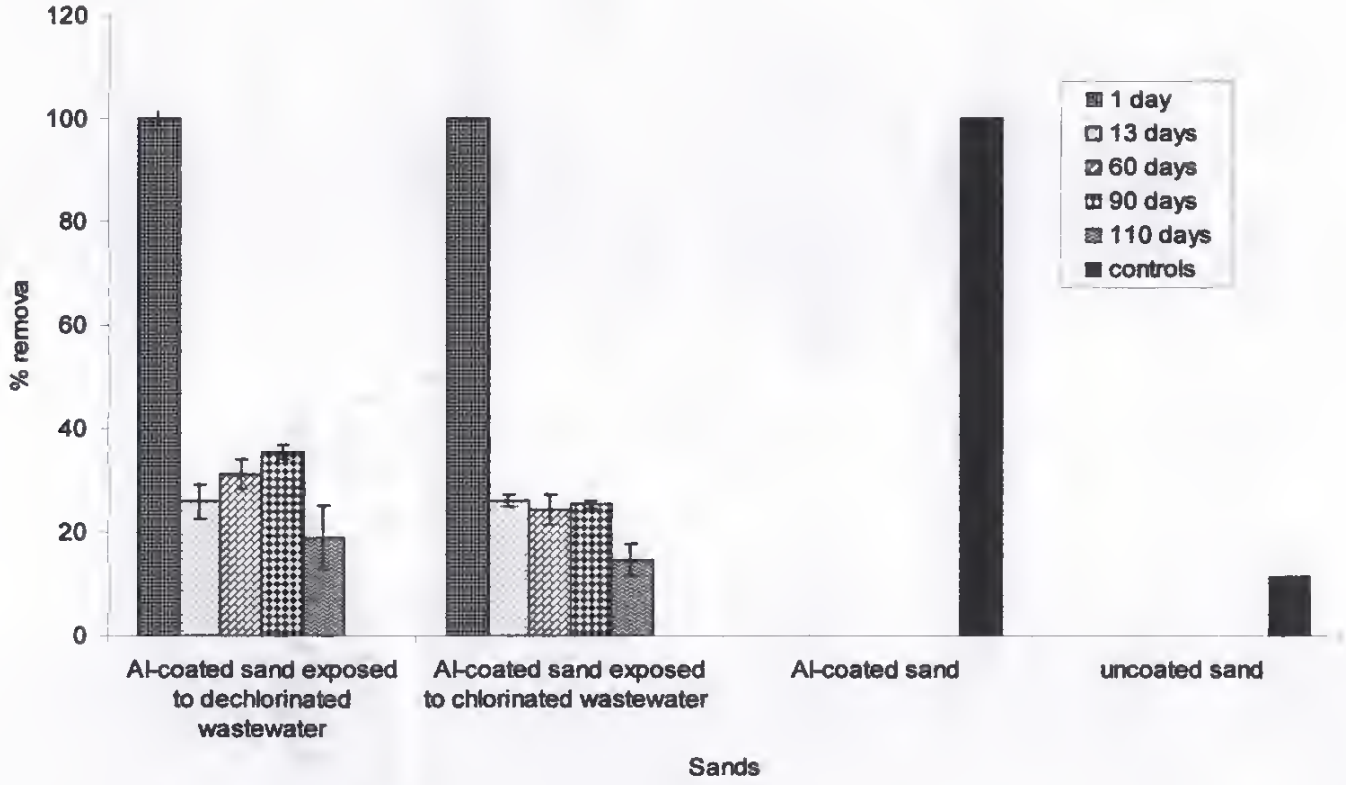

Fig. 11. MS2 removal by batch experiment

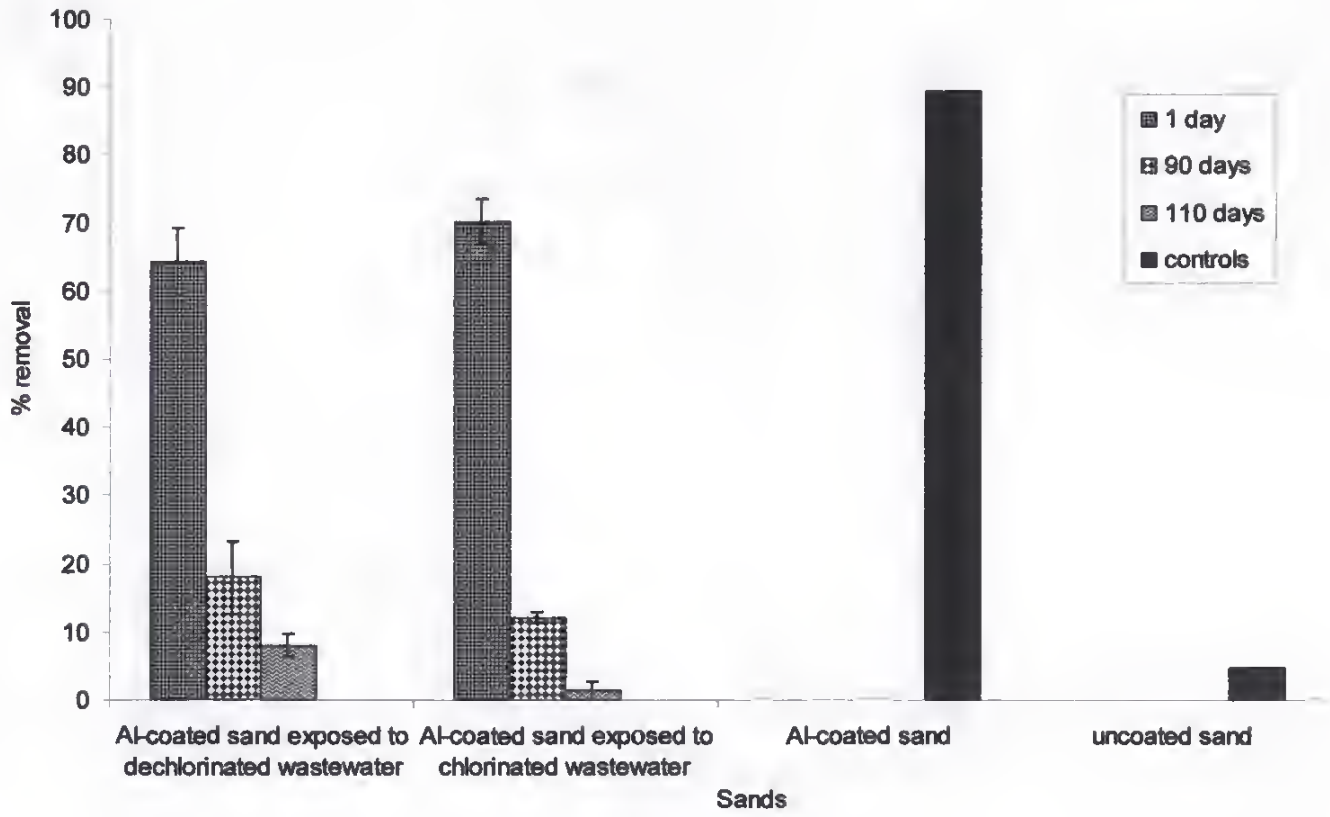

Fig. 12. PRD1 removal by batch experiment

DISCUSSION

Z. ramigera was found in wastewater treatment plants and some lakes. It was isolated from raw sewage, mixed liquor suspension and lake water by using m-toluic acid isolation medium combined with trimethoprim or sulfadiazine, which were more efficient than m-toluic acid isolation medium alone for isolation of *Z. ramigera* from natural environments.

Detection of *Z. ramigera* from natural environments is greatly simplified by using antibodies specific for the neotype strain of *Z. ramigera* 106 as the primary antibody, and different conjugates of goat anti-rabbit IgG which are commercially available as the secondary antibody. The cells and extracellular polymer of *Z. ramigera* were visualized in several ways using immunological procedures. These include using: A. FITC-labeled secondary antibody and fluorescence microscopy; B. biotin-conjugated secondary antibody and light microscopy; and C. gold-labeled antibody and SEM. The advantage of immunofluorescence procedures is that they are fairly easy to use. However, autofluorescence may be a problem with some samples. The enzymatic procedures are also fairly simple and can be done with a light microscope. Using gold-labeled antibody and SEM permits observation at higher magnifications than with light or fluorescence microscopy. Observations using backscatter electrons show where the antibody is localized, since only heavy metals, such as the gold of the secondary antibody, can cause back scatters.

Previously, the FITC-labeled 16S rRNA oligonucleotide probe against *Z. ramigera* 106 could also be used to detect *Z. ramigera* in natural samples, as was shown in a previous study by Rossello-Mora et al.(1995). However, a reduced content of intracellular 16S rRNA in less active cells, and limited penetration of the probe may be problem with this procedure. These potential problems could be overcome by using immunological methods. Indirect immunoassay methods are also technically simpler and more time saving than the detection method using the fluorescein-labeled 16S rRNA oligonucleotide probe.

PCR/RT-PCR has been used as a sensitive method to detect bacteria or viruses from environmental samples. By using the primers specific for *Z. ramigera* 106 16S rRNA, RT-PCR was used as another alternative method for detection of *Z. ramigera* from natural environments. There were negative reactions when 16S rRNA isolated from *Duganella zoogloeoides*, or from '*Z. ramigera*' 1-16-M was used as template for RT-PCR reaction. However, the same length of DNA fragments (619 bases) was produced when 16S rRNA isolated from *Z. ramigera* 106, *Z. ramigera* isolated from raw sewage, or from biofilm developed over raw sewage supplemented with phenol was used as template for RT-PCR. Therefore, the current procedure is reliable for *Z. ramigera* detection.

Immunological methods detect both dead and viable microorganisms as long as the presence of the antigen. Since rRNA is a dominant cellular macromolecule and related to viability of the cells, RT-PCR by using rRNA as template can be used to

detect only viable microorganisms. Generally, reproducibility and reliability of RNA extraction is important for reliable information obtained by RT-PCR, especially during studies of microbial community in dirty environments (Muyzer and Ramsing, 1995). Therefore, RT-PCR combined with the use of immunological methods is preferred for detection and understanding of *Z. ramigera* in natural environments.

Because it has been observed in association with biological flocs and biofilms in wastewater treatment plants, *Z. ramigera* has been considered to be an important microorganism in the wastewater treatment processes. However, studies on the number of bacteria capable of forming finger-like zoogloeae showed that these bacteria were a minor portion of the population of the samples from waste water (Williams and Unz, 1983). Higher numbers of *Z. ramigera* were found in activated sludge flocs by Rosello-Mora et al. (1995). These workers found that 10% of the cells in flocs from an aeration basin from one plant reacted with an oligonucleotide probe specific for *Z. ramigera* 16S rRNA.

In the current study, enrichment cultures using phenol were used to determine the MPN of *Z. ramigera* in wastewater and environmental samples. The presence of *Z. ramigera* was determined by observing the presence of typical finger-like projections. These were confirmed using immunological procedures. In some cases, fluorescence techniques revealed the presence of bacteria in typical zoogloeal projections that were obscured by other bacteria when the samples were observed using phase contrast microscopy. Using these procedures, the numbers of *Z. ramigera* in mixed liquor

suspended solids was closer to the value obtained using the FITC-labeled 16S rRNA oligonucleotide probe (Rossello-mora et al., 1995) than to the values obtained using microscopic examination of fingered zoogloeae (Williams and Unz, 1983).

The use of enrichment cultures and immunological procedures to enumerate *Z. ramigera* was meant to demonstrate their potential for studying the distribution of *Z. ramigera* in wastewater and environmental samples. Too few samples were taken for the numbers to be considered definitive. However, the numbers do show some interesting trends. It appears that *Z. ramigera* is more numerous in mixed liquor suspended solids. They are relatively fewer in raw sewage and in solids undergoing aerobic digestion. Besides wastewater treatment plants, *Z. ramigera* was also found in eutrophic, mesotrophic and some oligotrophic lakes. However, *Z. ramigera* was not found in one lake studied with very low total nitrogen content (Lake Sheelar, Table 5).

SEM observations of gold-coated *Z. ramigera* 106 indicated the presence of fibrils within and on the surface of the zoogloeal projections. It is likely that these fibrils provide the structure for the observed zoogloeal projections. Similar structures were found on the sand from the column that had sewage with phenol passed through it. Structures resembling zoogloeae were also observed in the biofilm from a denitrifying filter by Sich and Rijn (1997). Since natural zoogloeal finger-like projections reacted with antibodies against *Z. ramigera* 106 cells or extracellular polymer, the natural zoogloeae were also antigenically similar to laboratory culture.The antigenic similarity between natural finger-like projection and the laboratory culture suggests that the

material surrounding natural finger-like projections are chemically similar to that surrounding *Z. ramigera* 106. This extracellular material was previously shown to be a mucopolysaccharide associated with flocculation (Farrah and Unz, 1976; Tezuka, 1973; Unz and Farrah, 1976b) and could be recovered from laboratory cultures of *Z. ramigera* and from mixed liquor suspended solids.

Unlike some extracellular polymer-producing bacteria whose clones on nutrient agar plate are mucoid or butyrous, *Z. ramigera* clones on the agar plate are gelatinous, dry and hard. It was found that zoogloeal projections can even resist protozoan attack in activated sludge flocs (Farrah, 1974). Previous knowledge and current information obtained by GC/MS analysis of the extracellular polymer produced by *Z. ramigera* indicated that the predominance of carbohydrate, especially amino sugars. Generally, a bacterial extracellular polymer not only provides a major source of biomass as an alternation energy source, but also provides structural rigidity and a defensive barrier to invading pathogens. It was observed that the finger-like projection structure of *Z. ramigera* can even resist protozoan attack in activated sludge flocs (Farrah, 1974).

It was found that bacterial cells in biofilm are usually more resistant to environmental stress and antibacterial agents than planktonic cells (Marshall et al., 1989; Ophir and Gutnick, 1994; Brown et al., 1995). In the case of chlorination of biofilm bacteria, it was suggested transport of chlorine to biofilm bacteria surface might be an important rate-limiting step (LeChevallier et al., 1988 a,b; Huang et al., 1995). Free chlorine is known to react with a wide variety of compounds, including

polysaccharide. Therefore, besides providing a physical barrier reducing the ability of chlorine to approach the cell membrane, free chlorine might be consumed by reaction with extracellular polymer before it can fully penetrate the biofilm surface (LeChevallier et al., 1988 a,b). In this study, it was found that *Z. ramigera* extracellular polymer had protected the cells from chlorine inactivation. *Z. ramigera* extracellular polymer might either prevent the direct contact of chlorine with the cells or react with chlorine.

Biofilm development on the filter media enhances removal of bacteria and fine particles from water (Schuler et al., 1991; Banks and Bryers, 1992; Drury et al., 1993; Rittmann and Wirtel, 1991; Sprouse and Rittmann , 1990). In this study, the loss in zeta potential appeared to be more important than biofilm development on the surface of the aluminum coated sand after long term exposure to dechlorinated wastewater. Therefore, virus removal by the sand was greatly reduced after wastewater exposure. As with biofilm development, organic and inorganic materials in wastewater also 'blocked' favorable sites for virus removal. Thus, the coated sand exposed to chlorinated wastewater also reduced virus removal.

The results of this study can be summarized as follows:

1. Isolation media for the recovery of *Z. ramigera* from natural environments can be improved by adding the antibiotics trimethoprim and sulfadiazine.

2. SEM analysis of finger-like projections of laboratory cultures of *Z. ramigera* shows the presence of fibrils running along the length of the zoogloeal projections.

3. Immunological assays showed that both the cells and extracellular polymer of natural finger-like projections are antigenically similar to those of *Z. ramigera* 106.

4. The major components of the extracellular polymer surrounding *Z. ramigera* are galactosamine and another unidentified amino sugar.

5. The extracellular polymer protects *Z. ramigera* cells from inactivation by chlorine.

6. *Z. ramigera* can be found in all stages of wastewater treatment, but are present in the highest percentage in mixed liquor samples. *Z. ramigera* can also be found in eutrophic, mesotrophic and some of oligotrophic lakes, but not in the lake with low nitrogen content.

7. Exposure of modified sand to wastewater decreases its ability to adsorb viruses in water.

8. Part of the biofilm that develops on sand exposed to wastewater containing phenol have structures that resemble finger-like projections of *Z. ramigera.*

APPENDIX
SEQUENCE OF *Z. RAMIGERA* (ATCC 19544) 16S RRNA

ORIGIN

1 agagt t tga tnn tggct cagattgaacgctggcggcatgc t t tacacatgcaag tcgaac
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121 gccg tg taa tggggga taacg tagcgaaag ttacgctaataccgca tacgccctgagggg
181 gaaag tgggggaccgcaaggcc tcacg t tatacgagcggccga tgtcggat tagctagtt
241 gg tggggtaaaggcc taccaaggcgacga tccg tagcgggt ctgagaggatgatccgcca
-->P1
301 cac tgggac tgagacacggcccagac tcctacgggaggcagcagt ggggaatt ttggaca
361 at gggggcaaccc tga tccagccatgccgcg tgagtgaacaaggcct tcgggt tgtaaag
421 ctct t tcagg tggaaagaaa tcgcatc tt ttaatacaggg tg tgga tgacgg tacca tca
481 gaagaagcaccggctaac tacg tgccagcagccgcggtaatacg taggg tgcgagcg tta
541 a tcggaa ttac tgggcgtaaagcg tgcgcaggcggt tatg taagacaga tgtgaaa tccc
601 cgggc tcaacctgggaactgcgt t tgtgac tgc**ataactagagtacggcag**agggaggtg
661 gaa ttccgcg tg tagcag tgaaatgcgtagagatgcggaggaacaccgatggcgaaggca
721 gcctcc tgggccag tactgacgctca tgcacgaaagcg tggggagcaaacaggat tagat
781 accc tgg tag t ccacgccct aaacgatgtcaactagt tgt tcgg tgaggagac tcattga
P2 <-----gatcaacaagccac tcc tc tgagtaa
841 g taacgcagc taacgcgtgaagt tgaccgcctggggag tacggccgcaaggt taaaactc
901 aaaggaat tgacggggacccgcacaagcggt ggatgatg tggat taat tcgatgcaacgc
961 gaaaaacct tacc taccct tgacatgccaggaact tgccagaga tggct tgg tgcccgaa
1021 agggaacc tggacacaggtgc tgca tggctg tcgtcagc tcgtg tcgtgagatgt tgggt
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1141 a tgagac tgccgg tgacaaaccggaggaaggtggggatgacgtcaagtcctca tggccct
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1261 tggagccaa tcccagaaagccga tcgtag tccggat tggagtc tgcaac tcgactcca tg
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1381 gtacacaccgcccg tcacaccatgggag tggggt t taccagaagt aggtagct taacct t
1441 cgggagggcgct taccacggtgagct tcatgac tgggg tgaag tcgtaacaaggtagccg
1501 tatcggaaggtgcggctggatcacctcctt
//

Note: the bold letters indicate the sequence complementary to 16S rRNA oligonucleotide probe used previously. Underlined letters indicate the primers used in RT-PCR reaction.

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BIOGRAPHICAL SKETCH

Fuhua Lu was born on June 4, 1969, in Shanxi province, P. R. China. She received a Bachelor of Science degree in biology in July, 1991, from Wuhan University, P. R. China. After graduation, she went to the Institute of Applied Ecology, Academia Sinica, and received a Master of Science in microbiology in July,1994. In fall of 1994, she enrolled in the Ph.D program in the Department of Microbiology and Cell Science at the University of Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Samuel R. Farrah

Samuel R. Farrah, Chair
Professor of Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

K.T. Shanmugam

Keelnatham T. Shanmugam
Professor of Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

E M Hoffmann

Edward M. Hoffmann
Professor of Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ann C. Wilkie

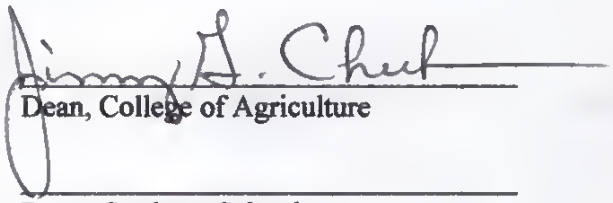
Ann C. Wilkie
Assistant Professor of Soil and Water Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ben L. Koopman
Professor of Environmental Engineering Sciences

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1998



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